

GENERATION OF SEXUAL AND SOMATIC HYBRIDS IN ACID CITRUS FRUITS

By

ZENAIDA JOSEFINA VILORIA VILLALOBOS

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2003

Copyright 2003

by

Zenaida Josefina Viloria Villalobos

This dissertation is dedicated to my darling mother Olivia and to the memory of my beloved father Dimas, and to my sisters Celina, Doris, Celmira, and Olivia, and brothers Dimas, Silfredo and Alejandro, with love.

ACKNOWLEDGMENTS

This work was completed with the generous collaboration of many people to whom I will always be grateful. First I wish to thank my supervisor Dr. Jude Grosser, for his guidance, suggestions, and financial assistance during the last period of my studies. I also want to thank the University of Zulia and Fondo Nacional de Ciencias, Tecnologia e Innovación for giving me the opportunity to do my doctoral studies. I thank very much Dr. Renee Goodrich, Dr. Frederick Gmitter, Dr. Michael Kane and Dr. Dennis Gray for being members of my committee and for their contributions to this work. Thanks go to Dr. Glem Wright (University of Arizona) for making it possible to generate more lemon progenies in this study. I appreciate very much the supervision and help in completing the canker screening study from Dr. Graham, Diana Drouillard and Diane Bright. I thank very much Dr. Ramon Littell and Belkys Bracho for their assistance on the statistical analysis of my experiments. Thanks go to the Division of Plant Industry (Lake Alfred, FL), particularly to Mrs. Vicky Fisher and Mr. Mike Kesinger, who helped me by taking care of my seed progenitors and providing budwood. I am very grateful to Millica Calovic, who not only gave me suggestions and all the help I needed, but also cheered me up when I was down. I appreciate the unconditional support from Beatriz Nielsen, who made my laboratory work easier. I am also very grateful to Jenny Noling for her opportune help in my busiest times; J.L. Chandler for being a good listener and helper in my field and lab work and proofreading my manuscripts; Orrina for taking care of my hybrids in the greenhouse ; Patricia, who took care of my contaminated cultures and

shared her nutrient media and good humor with me; Cassia for giving me priority in the use of laboratory equipment and instruments; Oscar, Anantha and Wen Wu for their suggestions and physical help when I was weak; and Ahmed who shared his computer expertise to make my presentations better. I thank Gretchen Baut for her help and assistance on photography and Pamela Russ who graciously provided all requested help in library matters.

There were people that did not participate directly in my work but they made my life easier and more enjoyable. I will always be thankful to Julia Meredith and Renato Inserra for helping me to settle down and learn many things about American culture. I want to thank the following people for their friendship and support: Elisabeth, Mukkades, Angie, J.P., Marty, Fahiem, John Jiffon, Mondal, Azza, Elio, Joaquin, Octavio, Brandon, Raul, Hugo, Jose Carlos. I am also grateful to Maritza, Alba and Umpika for hosting and helping me at all times. I finally offer thanks to my beloved family for their support. Special thanks to my sister Celina for having done good work taking care of my errands in Maracaibo.

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	xi
ABSTRACT	xii
 CHAPTER	
1 INTRODUCTION	1
General Introduction	1
Review of Literature	4
2 IMPROVEMENT OF ACID CITRUS FRUIT CULTIVARS VIA INTERPLOID HYBRIDIZATION	17
Introduction	17
Materials and Methods	20
Results	23
Discussion	27
3 INDUCTION OF <i>IN VITRO</i> GERMINATION AND SEEDLING DEVELOPMENT OF IMMATURE EMBRYOS RESULTING FROM INTERPLOID HYBRIDIZATION IN ACID CITRUS FRUITs	43
Introduction	43
Materials and Methods	45
Results	48
Discussion	51
4 GERMINATION INDUCTION OF ABORTED TRIPLOID HYBRID MANDARIN EMBRYOS	62
Introduction	62
Materials and Methods	63
Results	65
Discussion	65

5	A STOMATAL INOCULATION METHOD FOR SCREENING TRIPLOID HYBRIDS OF LAKELAND LIMEQUAT FOR RESISTANCE TO ASIATIC CITRUS CANCKER (<i>Xanthomonas axonopodis</i> pv. <i>citri</i>)	71
	Introduction.....	71
	Materials and Methods	74
	Results.....	77
	Discussion.....	79
6	SOMATIC HYBRIDIZATION between <i>Citrus limon</i> (L.) BURM. F. AND OTHER CITRUS SPECIES: REGENERATION OF PROTOPLAST-FUSION PRODUCTS	86
	Introduction.....	86
	Materials and Methods	88
	Results.....	92
	Discussion.....	95
7	SUMMARY AND CONCLUSIONS	103
APPENDIX		
A	COMPOSITION OF THE EME MEDIUM	107
B	COMPOSITION OF RMA MEDIUM	108
C	COMPOSITION OF 0.6M BH3 NUTRIENT MEDIUM	109
D	COMPOSITION OF SUCROSE AND MANNITOL SOLUTION GRADIENT ...	110
E	COMPOSITION OF A AND B SOLUTIONS	111
F	COMPOSITION OF H-H EME MEDIUM.....	112
G	COMPOSITION OF B+ MEDIUM	113
H	COMPOSITION OF DBA3 MEDIUM.....	114
	LIST OF REFERENCES.....	115
	BIOGRAPHICAL SKETCH.....	125

LIST OF TABLES

<u>Table</u>	<u>page</u>
2.1 Pollen viability according to acetocarmine staining assay of staminate progenitors utilized in the crosses of the 2000 and 2001 seasons.....	32
2.2 Fruit set and seed number of acid citrus progenies obtained from diploid x tetraploid crosses completed in the 2000 season.....	33
2.3 Plant recovery and ploidy level of acid citrus progenies generated from diploid x tetraploid crosses performed in the 2000 season.....	34
2.4 Fruit set, seed and embryo number of acid citrus progenies from diploid x tetraploid crosses completed in the 2001 season.....	35
2.5 Lemon fruit set, seed and embryo number obtained from diploid x tetraploid crosses completed in the 2001 season.	36
2.6 Plant recovery and ploidy level of acid citrus progenies derived from diploid x tetraploid crosses completed in 2001.	37
2.7 Plant recovery and ploidy level of lemon progenies generated from diploid x tetraploid crosses in 2001 season.	38
2.8 Fruit set, seed number and ploidy of rescued embryos from tetraploid x diploid crosses made in the 2000 season.	39
2.9 Fruit set, seed number and ploidy of rescued embryos obtained from tetraploid - x-diploid crosses completed in the 2001 season.	40
2.10 Fruit set, seed and embryo number of lemons from diploid x tetraploid crosses completed in the 2002 season.....	41
2.11 Plant recovery and ploidy level of lemon progenies generated from diploid x tetraploid crosses completed in 2002.	41
3.1 Developmental stages of lemon embryos obtained from diploid x tetraploid crosses 105-110 days after pollination corresponding to the 2001 season.....	56
3.2 Developmental stages of acid-citrus fruit embryos obtained from diploid x tetraploid crosses in the 2001 season.	57

3.3	Developmental stages of embryos obtained from tetraploid x diploid crosses 90 days after pollination in the 2001 season.	57
3.4	Analysis of variance of percentage germination of small embryos rescued from diploid-x-tetraploid crosses completed in the 2001 season.	58
3.5	Mean percentages of small embryo germination and normal seedling recovery from small embryos.....	58
3.6	Analysis of variance of normal seedling percentage recovered from small embryos obtained from diploid-x-tetraploid crosses.....	59
3.7	Analysis of variance of percentage germination of immature cotyledonary embryos obtained from diploid-x-tetraploid crosses.....	59
3.8	Mean of germination and normal seedling percentages of immature cotyledonary embryos from diploid-x-tetraploid crosses.	60
3.9	Mean of germination percentage of immature cotyledonary embryos determined by the sucrose-cross interaction.	60
3.10	Analysis of variance of normal seedling percentage recovered from immature cotyledonary embryos-obtained from diploid-x-tetraploid crosses.	61
3.11	Analysis of variance of plant height of plants recovered from rescued-immature embryos generated in diploid-x-tetraploid crosses.....	61
3.12	Analysis of variance of root length of plants recovered from rescued-immature embryos generated in diploid-x-tetraploid crosses.....	61
4.1	Analysis of variance of germination percentage of rescued embryos from interploid crosses of LB8-9 mandarin x 'Nova' mandarin + 'Succari' orange.....	69
5.1	Mean of lesion number/inoculation site induced by stomatal inoculation of <i>Xanthomonas axonopodis</i> pv. <i>citri</i> on 'Lakeland' lime hybrids in four repeated assays.....	82
5.2	Spearman's rank order correlation coefficients among genotype rankings according to lesion number/inoculation site after fifteen days of stomatal inoculation of <i>Xanthomonas axonopodis</i> pv. <i>citri</i>	83
5.3	Mean of bacterial population/ lesion induced by stomatal inoculation of <i>Xanthomonas axonopodis</i> pv. <i>citri</i> on 'Lakeland' limequat hybrids in two repeated assays.	84
6.1	Mean of embryo clusters and frequency of polyploids regenerated from 'Santa Teresa' lemon protoplast fused with several leaf donors and initially grown on different nutrient media.....	98

6.2	Mean of embryo clusters and frequency of polyploids regenerated from 'Lapithiotiki' lemon protoplast fused with different leaf donors and initially grown on different nutrient media.....	99
6.3	Effect of protoplast fusion techniques on protoplast regeneration and polyploid frequency of 'Lapithiotiki' lemon + 'Etrog' citron.....	99

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
2.1 Pollen viability assay using acetorcarmine staining.....	42
4.1 Mean of percentage germination of aborted LB8-9 mandarin embryos influenced by the GA- sucrose interaction.....	70
5.1 Phenotype of ACC lesions developed two weeks after inoculation.....	85
6.1 Amplified DNA banding patterns using the random primers C64.....	100
6.2 Leaf morphology of 'Santa Teresa' lemon + <i>C. ichangensis</i> somatic hybrid.....	100
6.3 Amplified DNA banding pattern using W-09 primer.	101
6.4 Leaf morphology of 'Lapithiotiki' lemon + 'Lakeland' limequat and 'Lapithiotiki' lemon + 'Chinotto' sour orange somatic hybrids and their progenitors.....	101
6.5 Amplified DNA banding pattern using U-18 primer.	102
6.6 Leaf morphology of 'Lapithiotiki' lemon + 'Etrog' citron somatic hybrids and their progenitors	102

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

GENERATION OF SEXUAL AND SOMATIC HYBRIDS IN ACID CITRUS FRUITS

By

Zenaida Josefina Vilorio Villalobos

May 2003

Chairman: Dr. Jude W. Grosser

Major Department: Horticultural Sciences

Interploid hybridization was completed between diploid progenitors ('Key' lime, 'Lakeland' limequat, Palestine sweet lime, 'Etrog' citron and seven lemon varieties) and five allotetraploid somatic hybrids and two autotetraploids as pollen progenitors. A few reciprocal crosses were also carried out. Thirty five parental cross combinations were accomplished in 2000, 2001 and 2002. The breeding targets were seedlessness, cold tolerance, and disease resistance. Immature embryos were rescued and cultured on two nutrient media, with two sucrose concentrations. Germination of globular-early cotyledonary embryos was significantly ($P<0.05$) affected by genotype and medium. Gamborg's B5 medium induced 82.89% germination. However, 11-40% of germinated embryos developed into normal plants with differences among crosses. Early cotyledonary embryos germinated and developed into normal plant at a higher rate on either EME or Gamborg's B5. Approximately 650 triploids were transferred to soil. Additionally, the effect of gibberellic acid (GA), medium and sucrose as well as their interactions were evaluated on aborted triploid mandarin embryos (LB8-9 mandarin

hybrid x (Nova mandarin hybrid + Succari orange)). Germination of mandarin embryos was significantly ($P < 0.0001$) improved by Gamborg's medium (62.86%) and the addition of sucrose (204.50 mM) and GA ($4.54 \mu\text{M}$).

'Lakeland' limequat progenies were screened for resistance to Asiatic citrus canker (*Xanthomonas axonopodis* pv. *citri*). Nine triploids, two tetraploids, five progenitors and susceptible and resistant standards were inoculated via stomata using a syringe without needle. Significantly strong correlations among genotype rankings for lesion number and bacterial population were obtained in repetitive assays. Hybrids from 'Key' lime + 'Valencia' orange and 'Hamlin' orange + 'Femminello' lemon showed promising levels of resistance.

Somatic hybrids were generated between 'Lapithiotiki' and 'Santa Teresa' lemons as cell suspension parents and the leaf parents: *Citrus medica*, *C. aurantium* cv. 'Chinotto', *C. micrantha*, *C. ichangensis*, and 'Lakeland' limequat hybrid. The purpose was to increase the breeding germplasm of acid citrus fruit cultivars. Furthermore, the media BH3, EME and a 1:1 mixture of BH3 and EME were used to induce protoplast embryogenesis. The lowest regeneration was observed when EME was used as initial nutrient medium for protoplast culture in some parental combinations. The frequency of tetraploid embryo clusters was higher in 'Lapithiotiki' fusions. Efficiency of allotetraploid somatic hybrid regeneration was enhanced by modifying protoplast fusion protocol in protoplast, medium and solution volumes and incubation time as well as the substitution of Petri dishes by culture tubes.

CHAPTER 1 INTRODUCTION

General Introduction

Commercial acid citrus fruit is primarily represented by lemon (*Citrus limon* (L.) Burm. f.) in world production, followed by acid limes. Lemon varieties are classified in three major groups: Femminello, Sicilian and Verna, and each group has many varieties. 'Mexican' ('West Indian' or 'Key' lime) is the major variety of a true acid lime (*Citrus aurantifolia* Swing.), whereas 'Tahiti' ('Persian' or 'Bearss') lime is a triploid hybrid of unknown origin, identified Tanaka as *Citrus latifolia* Tan. Lemon and lime fruits have different morphologies and flavors, although it can be difficult to separate some varieties.

Worldwide production of lemon and lime has been increasing, reaching 11,038,711 MT in the 2001-2002 season. Argentina is the leading lemon producer followed by Spain, the United States, Italy and Turkey (Florida Agricultural services, 2002a). Mexico is the major producer of 'Mexican' lime, with 70,000 bearing ha (Medina et al., 1995). Acid citrus fruit production is approximately 10% of total citrus world production, lemon-and-lime production has a significant trade and impact in the soft drink and perfume industries.

Acid citrus fruit production is limited by adverse climatic conditions, and diseases and pests that vary from country to country. In humid climatic conditions such as in Florida, fruit quality is low owing to fungal diseases and the development of larger fruit (Sinclair 1984). Sporadic freezes also limit lemon production. Limes are tropical; nonetheless they are occasionally grown in warm and humid regions of the subtropics.

Acid citrus fruit varieties are susceptible to diseases that are minimally or not controlled with pesticides, for example citrus tristeza virus (CTV) and Asiatic citrus canker (*Xanthomonas axonopodis* pv. *citri*) (ACC). CTV is the most economically important viral citrus disease worldwide. Symptoms are variable; stunting, stem pitting, leaf cupping, vein-clearing, chlorosis and tree decline (Garnsey and Miyakawa 1988).

Asiatic citrus canker is a bacterial disease that causes necrotic erupted lesions on leaves, stem and fruit. Severe infection induces defoliation, dieback, premature fruit dropping, fruit damage and tree decline (Schubert 2001; Koizumi 1985). 'Mexican' lime is highly susceptible to ACC. Currently, 88% of the lime acreage has been removed as the result of an eradication program being conducted in Florida since 1995 (Florida Agricultural Services, 2002b).

Most Italian lemon varieties are susceptible to 'mal secco', a systemic fungal disease caused by *Phoma tracheiphilla*. The fungus penetrates through wounds and colonizes the xylem, causing dieback (Tusa et al. 1990; 2000). Witches' broom is another dangerous systemic disease, caused by a mycoplasma-like organism (*Phytoplasma aurantifolia*) that has been reported in acid lime grown in the Sultanate of Oman and the United Arab Emirates (Garnier et al. 1991). Breeding for disease resistance may solve such serious disease problems in acid citrus.

Furthermore, fruit quality is one of the main targets of acid citrus cultivar breeding programs. Seedless acid citrus fruit are preferred by fresh fruit market, while acid yields, high oil quality and oil content are the main desirable attributes for the processing market. Indeed, Ruberto et al. (1997) noticed that when selecting for fresh fruit quality, high standards for industrial quality could also be reached. Fruit essential oil

composition in lemon triploid hybrids generated through interploid hybridization showed higher limonene and myrcene (Gazea et al. 1996) and quantitative and qualitative differences in Clementine triploid hybrids (Ruberto et al. 1997).

Leaf essential oil composition has been analyzed in somatic and sexual hybrids. 'Milam' lemon + 'Femminello' lemon somatic hybrid was characterized by a lower hydrocarbon fraction and an increase of oxygenated components compared to the progenitors (Alonzo et al. 2000). A citrus cybrid of 'Valencia' sweet orange and 'Femminello' lemon showed a higher percentage of bethacaryophyllene and higher quality and quantity of the esteric fraction (Fatta del Bosco et al. 1998). These reports indicate that quality and quantity of leaf and fruit essential oils can be genetically improved through sexual or somatic hybridization using outstanding progenitors.

Genetic improvement of current acid fruit cultivars is a worthwhile effort to face the aforementioned constraints and to enhance fruit quality. Sexual hybridization faces some major constraints including long juvenility periods, polyembony, sexual sterility, cross incompatibility and inbreeding depression (Vardi 1992). Since genetic variability is essential in breeding programs, selected citrus allotetraploid somatic hybrids can be a source of new genes and combinations of genes or alleles to enrich future acid citrus fruit progenies. Hybridization via interploid crosses offers a feasible approach to improve citrus fruit quality and introgress other desirable traits. The success of interploid crosses in polyembryonic species is determined by the opportune rescue of immature zygotic embryos, and an adequate nutrient medium for embryo germination *in vitro*.

The principal objectives of this study were:

1. To generate seedless triploid progenies of acid citrus fruit with substantial genetic variability and potential for improved fruit quality, cold hardiness and improved disease resistance through interploidy hybridization (Chapter 2).
2. To optimize protocols to recover immature embryos generated from interploidy hybridization (Chapter 3 and 4).
3. To generate allotetraploid somatic hybrids with promising genome combinations for further use as progenitors in breeding programs for acid citrus fruit improvement (Chapter 5).
4. To validate a canker resistance screening protocol by applying a modification of the Gottwald-Graham methodology on triploid hybrids of 'Lakeland' limequat (Chapter 6).

Review of Literature

Citrus Breeding

Citrus breeding programs have been hampered by factors such as sterility, sexual incompatibility, polyembryony, long juvenile period and the lack of information about the inheritance of important traits (Grosser and Gmitter 1990).

The citrus fresh fruit market demands high quality seedless fruit. Seedless progenies have been generated through different procedures including interploidy hybridization between diploid and tetraploid cultivars in either direction (Cameron and Soost 1969; Esen and Soost 1972b; Cameron and Burnett 1978; Oiyama and Kobayashi 1990); selection of spontaneous triploid embryos arising from diploid x diploid crosses (Ollitrault et al. 1996); and gamma irradiation to induce seedless mutants (Hearn 1984; Hensz 1977). In Australia, the citrus breeding program is attempting to produce seedless fruit through diploid x diploid hybridization using parents with genes for parthenocarpy, male/female sterility, and pollen incompatibility (Sykes and Lewis 1996; Koltunow et al. 2000).

Interploid hybridization has been routinely applied in citrus despite the difficulties associated with the high incidence of embryo failure and underdeveloped seeds. Studies on seed development and cytology from diploid x tetraploid crosses suggested that the ploidy ratio of the chromosome number between zygotic embryo and endosperm (3:4) affects the physiological interactions between them. Consequently the endosperm aborts inducing embryo failure (Esen and Soost 1973).

The endosperm balance number hypothesis has been proposed to explain the basis for normal seed development in intra- and inter-specific crosses. It states that each species has a genome-specific ploidy, which must be 2:1 of maternal-paternal ratio in the hybrid endosperm itself (Carputo et al. 1999). The endosperm balance number hypothesis establishes that the different results obtained in interploid crosses, diploid by tetraploid and the reciprocal, are based on the ratio $2x \times 4x$ $4x \times 2x$ 1:1 or 4:1 of maternal-paternal ratio, respectively, in the endosperm (Johnston et al. 1980).

Selection of strictly or highly sexual diploid seed parents contributes to triploid hybrid production even though the fruit set is low (Starrantino and Reforgiato Recupero 1981; Oiyama et al. 1981). However, a high number of hybrid tetraploids can also occur when a tetraploid pollen parent is chosen, reducing the proportion of triploids (Cameron and Soost 1969). Higher numbers of triploid hybrids were generated when the seed parent was a sexual tetraploid (Cameron and Burnett 1978). In those cases, the embryo-endosperm ratio is 3:5, which seems to be favorable for small but fully developed seed (Esen and Soost 1973). Unfortunately, polyembryony is a character present in most citrus species, including lemons and limes.

Efficiency of interploid hybridization has been enhanced through the incorporation of the embryo culture technique to the breeding system. Immature or aborted embryos are rescued and cultured on artificial nutrient medium under controlled environmental conditions to induce their development. Furthermore, interploid hybridization was originally limited owing to the scarce availability of autotetraploid parents (Oiyama and Kobayashi 1990; Esen and Soost 1972). Somatic hybrids are valuable breeding progenitors that can be utilized in interploid hybridization to generate seedless progenies (Grosser et al. 2000).

Embryo Culture

In plant breeding, embryo culture is a useful technique to recover hybrids from interspecific, intergeneric and interploid crosses that are not able to fully develop due to endosperm degeneration. This technique has also been applied to rescue embryos from abortion in early ripening peach (Ramming 1985) and grape (Ramming 1990) varieties. In those varieties, fruit develops faster than seed. Breeding cycle can also be shortened using embryo culture to break dormancy (Collins and grosser 19984).

The growth of immature peach embryos was improved by culturing perforated ovules on media with 10% sucrose. Nutrient media did not affect embryo growth (Pinto et al. 1994). Optimum germination and high percentage of germinated embryo developing into plants was induced with woody plant medium (WPM) on peach excised embryos. Some peach genotypes responded better to higher salt media such as MS and C2d (Emershad and Ramming, 1994). *In vitro* embryo culture and ovule maturity determined the success of plant recovery from nectarine ovules germinated on Knop's medium (Chaparro and Sherman 1994). In any case, immature embryos or ovules culture

are successfully applied to rescue peach and nectarine embryos that otherwise would abort. Fine-tuned protocols need be developed for the particular genotype.

In muscadine grape, embryo recovery was affected by genotype, relative ovule maturity and the interaction of both factors (Gray and Hanger 1993). More zygotic embryos (35%) were recovered from older ovules (40 days after pollination). Genotype and embryo age also had a great influence on recovery of embryos from seed trace of 18 seedless grape genotypes. More plants were obtained from older embryos (42-70 days after past bloom) germinated better on Cain's basal medium containing 6% sucrose, while germinated embryo growth was induced on woody plant medium with lower sucrose content (Pommer et al. 1995). Nitsch and Nitsch's medium supplemented with activated charcoal, sucrose and plant growth regulators was also used for grape seed traces and excised embryos (Valdez and Ulanovsky 1997). Excised embryos germinated at higher rate (41.30%), however the cross direction affected germination due to difference in seed coat hardness.

Efficient production of triploid grapes from reciprocal crosses between diploid and tetraploid cultivars was possible by *in ovulo* embryo culture. Triploid hybrids were recovered in higher number (20-86%) when the seed progenitor was tetraploid, and the embryos were at advanced developmental stages (50-65 days after pollination). Germination medium was half strength MS salts, supplemented with IAA and GA (Yamashita et al. 1998).

Rescue of interspecific hybrids of *Carica papaya* was improved through a 5-day pre-incubation of 90-day-old embryos onto half-strength De Fossard's medium supplemented with GA₃, BA, NAA and sucrose (Magdalita et al. 1996). Non-haploid

embryos of Japanese persimmon in globular and torpedo stages germinated at very low rate (2%) on modified MS medium; however some pro-embryos were rescued and grown successfully (Sugiura et al. 2000). More research needs to be done to improve germination rate. After evaluation of several medium components, it was determined that globular, heart-shaped and torpedo stages of interspecific hybrids of *Actinidia* species needed a rich medium, with high osmotic concentration (3% sucrose and 5% mannitol) to germinate. High germination rates were induced on globular and heart-shaped embryos (60%) and torpedo (80%). Changes in medium components were done in accordance with embryo development (Hirsch et al. 2001). The success of this report was based in the evaluation of several medium supplements associated with pro-embryos germination such as high osmoticum, plant growth regulators and other addenda and with further seedling development.

Several nutrient media have been evaluated for citrus embryo culture. Globular zygotic embryos from *Citrus aurantium* x *Poncirus trifoliata* crosses failed to survive *in vitro*, whereas heart-stage embryos were efficiently cultured on modified White's medium supplemented with casein hydrolysate and sucrose. Germinated embryos were transferred onto a medium without casein hydrolysate and sucrose to induce further growth (Rangan et al. 1969). Supplementing Murashige and Tucker (MT) medium with high sucrose and GA concentrations and malt extract induced 76% germination rate of *Citrus aurantium* globular embryos (Carimi et al. 1998), whereas heart-staged embryos require lower GA levels and early cotyledonary embryos germinated well on malt.

Murashige and Tucker medium is frequently used to rescue citrus aborted embryos resulting from interploid crosses. It contains MS mineral salts and higher MS vitamin

concentrations. It also has 2,4-D or NAA, kinetin and GA₃ (optional) and 50g/L sucrose (Murashige and Tucker 1969).

Triploid hybrids were recovered from aborted monoembryonic seeds collected seven month after pollination using MT supplemented with malt extract and adenine (Oiyama and Kobayashi 1990), mandarin plant recovery was 95.50% of plated embryo (Oiyama et al. 1991). Low germination rate (25.60 %) was induced on monoembryonic Pummelo and polyembryonic tangerine embryos excised 90-100 days after pollination, when MT was supplemented with either GA or malt extract (Deng et al. 1996).

Embryo germination on MS was determined by cross direction. It was poor in embryos from 2X x 4X and high in the reciprocal (75%). Differences were also influenced by season and cross combination (Khan et al. 1996). Successful triploid recovery (89.55%) was reported for MS and different cross combinations (Starrantino and Reforgiato Recupero 1981).

In acid citrus fruits, MT medium containing malt extract was applied to induce germination of young lemon embryos obtained from interploid hybridization 100-105 days after pollination. Germination rate was not reported (Tusa et al. 1996). In citrus interploid hybridization, monoembryonic seed progenitors are preferred to polyembryonic ones, since large triploid progenies are rescued. In fact, most of the previously cited works have been performed using monoembryonic seed progenitors. Polyembryony in citrus seeds not only affects the zygote survival (Soares Filho et al. 1992) but also multiples the embryo rescue work. Embryo genotype and developmental stage as well as medium and medium supplements determined to a large extent the recovery of citrus triploid hybrids. The lack of information about triploid embryo rescue

in acids citrus fruit makes necessary to investigate factors related with the *in vitro* germination of immature embryos and the efficient production of triploid hybrids.

Somatic Hybridization

Somatic hybridization is a very useful approach for overcoming the sexual barriers associated with conventional breeding (Saito et al. 1991; Kobayashi et al. 1991; Grosser and Gmitter 1990). Somatic hybrids embody the complete nuclear genome of both parents; therefore they could be grown as amphidiploid plant varieties or breeding progenitors in further breeding for improving somatic hybrids exhibiting poor horticultural performance (Bajaj 1994).

Multinational citrus variety improvement programs have developed more than 200 somatic hybrids and several cybrids aimed at developing useful breeding tetraploids to be used in interploid hybridization (Grosser et al. 2000). Elite diploid citrus species are chosen to enrich tetraploid citrus germplasm. Important traits such as fruit color and size, flavor, 'peelability' and maturity date can be genetically improved to increase fruit quality (Grosser et al. 1998; Grosser et al. 1992a).

Regarding acid citrus fruit, some somatic hybrids are combinations of parental genomes that were produced to create tetraploid parents for lemon cultivar improvement. The targets were to introgress genes for tolerance to cold and 'mal secco'. To accomplish those goals, sweet orange cultivars were selected as sources of genes of interest, generating 'Valencia' sweet orange + 'Femminello' lemon (Tusa et al. 1990) and Hamlin' sweet orange + 'Femminello' lemon (Tusa et al.1992). Similarly, 'Milam' lemon (*C. jambhiri*) was fused with 'Femminello' lemon to produce new breeding parents for subsequent use in lemon improvement programs (Tusa et al. 1992). Somatic hybrids tolerant to 'mal secco' were obtained by fusing 'Murcott' tangor (tolerant

progenitor) with the susceptible Messina' lemon (*C. limon*). Tolerant hybrids were selected after regeneration of protoplast fusion co-cultured with fungus toxin (Deng et al. 1995).

Acid lime has also been used to produce tetraploid somatic hybrids: protoplasts of 'Key' lime (*Citrus aurantifolia*) and 'Valencia' sweet orange (*Citrus sinensis*) were hybridized to produce a new citrus allotetraploid breeding parent. The purpose was to generate new acid limes, with reduced seed number and larger fruit. Sweet orange is expected to provide genes for improved cold hardiness, resistance to lime anthracnose and tolerance to citrus tristeza virus (Grosser et al. 1989). Another allotetraploid somatic hybrid was produced using acid lime (*Citrus aurantifolia*) as leaf parent with *Citrus sudachi* Hort. Ex Shiraim, a Japanese acid fruit that is resistant to cold (Saito et al. 1991).

Intergeneric somatic hybrids and some more interspecific hybrids have been produced worldwide using acid lime as a progenitor (Grosser et al. 2000). New allotetraploid somatic hybrids that combine acid lime genomes with other citrus species genomes are needed to increase the genetic diversity for acid citrus fruit cultivar improvement.

Brief Description of Selected Breeding Progenitors

Diploid progenitors

'Mexican' lime has a short juvenile period. Its fruit is small, acidic, seedy and spherical. The rind has the distinct pungent aroma which typifies 'West Indian' and 'Persian' limes (Saunt 1990). It is susceptible to cold and CTV.

'Lakeland' limequat is a hybrid between *C. auratifolia* and *Fortunella japonica* released in 1923, however it lacked commercial importance. It is occasionally found as a

dooryard tree. Trees are more cold tolerant than lemon and lime (Hearn 1985). Its fruit is similar to that of 'Mexican' lime, but of lesser quality.

'**Etrog**' ('**Ethrog**') **citron** has ellipsoid to fusiform fruit, with a nipple ending in the persistent style. Rind is thick and fleshy with prominent oil glands containing distinctive essential oils (Swingle 1967; Young 1986).

C. limettioides Tan. ('**Palestine**' **sweet lime**) fruit are medium size, round to oblong. They are juicy, but insipid, with a very low acid content. Rind is thin, smooth and greenish, with a distinctive oil aroma (Young 1986).

C. ichangensis Swing is a spiny shrub, with small fruit and rough peel. It is the most-cold resistant species of the subfamily Aurantioidea (Swingle 1967).

C. aurantium cv. '**Chinotto**' is a mutation from sour orange that has low vigor and short inter-nodes with more or less dense and compact canopy, and is thornless. Fruits are small, oblate-to-round; the pulp is orange in color. It stands winter cold better than sweet orange. It was classified as another species (*Citrus myrtifolia* Rafinesque) (Swingle 1967).

C. micrantha Wester. belongs to the most primitive group of citrus species. Fruit is obovate to oblong-obovate, lemon, yellow, smooth peel, juicy, grayish and acid (Swingle 1967).

Lemons

'**Eureka**' is a variety that originated in Los Angeles, California, in 1858 from Italian lemon seeds (probably the 'Lunario'). Selection was done in 1877. Fruit is elliptical to oblong, rarely obovate, with moderately protruding mammilla at the apex, juicy, very acid, and cold sensitive.

‘Genova’ (Genoa) is of Italian origin, with more spherical fruit, a small pointed neck and nipple, juicy, acidic, a thin rind and variable seed content. It has similar quality to ‘Eureka’. The tree is thornless, of a smaller size than ‘Eureka’, with dense foliage and more-cold resistant (Saunt 1990).

‘Arancino’ is an Italian variety; its fruit is nearly globose, with a short nipple and thick rind, and is very seedy. Trees are compact, and hardy with small spines.

Femminello is the most commonly grown group of lemons in Italy, ever bearing, seedy, moderately high acidity, lower juice content, with fruit shape that is prolate spherical with moderately sized mammilla. **‘Santa Teresa’** is a selection of ‘Femminello Ovale’ that was free from mal secco in a devastated grove (Morton 1997).

‘Limonero Fino 49’ is the most popular lemon in Spain. It is precocious, seedy, with symmetrical fruit that is easy to de-green. It is similar to ‘Eureka’, ‘Lisbon’ and ‘Genova’.

‘Lisbon’ is of Australian origin through seed exported from Portugal before 1824. It was introduced in California in the 1870s. It is better adapted to climates other than Mediterranean and is more cold resistant than ‘Eureka’ (Saunt 1990). It has high quality fruit, with prominent mammilla. Fruit is similar to Eureka, with higher yield, but seedy.

‘Limoneira 8A’ is a selection from ‘Lisbon’ made in California.

‘Lapithiotiki’ is a lemon variety of unknown origin. The trees are vigorous, productive, thorny and tolerant to mal secco. Fruit quality is similar to ‘Eureka’.

‘Cavers’ is an outstanding ‘Lisbon’ selection with high vigor and productivity (Swingle 1967). Information about **‘Todo el Año’** lemon was not found.

Tetraploid progenitors

‘Key’ lime + ‘Valencia’ orange somatic hybrid was expected to produce a hybrid with more cold tolerance, resistance to lime anthracnose and tolerance to citrus tristeza virus than the ‘Key’ lime (Grosser et al. 1989).

‘Hamlin’ orange + ‘Femminello’ lemon somatic hybrid contains ‘Hamlin’ that is tolerant to mal secco, more cold hardy than lemon, and ripens earlier than ‘Valencia’ orange, and ‘Femminello’ that has a good field performance and produces high quality fruit (Tusa et al. 1992).

‘Valencia’ orange + ‘Femminello’ lemon hybrid combines the cold hardiness and high tolerance to mal secco of ‘Valencia’ with the high quality of ‘Femminello’ (Tusa et al. 1990).

‘Milam’ hybrid + ‘Femminello’ lemon hybrid combines Milam that is considered a hybrid of *Citrus jambhiri* Lush., with a lemon type fruit with Femminello; this hybrid is expected to be a good lemon breeding progenitor (Tusa et al. 1992).

‘Valencia’ orange + Rough lemon hybrid was attempted to produce a vigorous rootstock, with improved cold hardiness and citrus blight tolerance (‘Valencia’ orange) (Grosser et al. 1992). Rough lemon has a lemon odor and flavor. It is supposed to be a hybrid of lemon and citron (Morton 1987).

‘Giant Key’ lime is an autotetraploid with fruit twice as large as fruit of diploid ‘Key’ lime. It bears year-round and has fruit flavor and aroma identical to diploid ‘Key’ lime fruit. It is a very attractive pot tree (Barrett 1992).

Asiatic Citrus Canker

The Asiatic strain or canker A is a group of strains initially found in Asia that causes the most virulent and widely spread disease (Gottwald, 2002). Infection occurs

when immature tissues are water-soaked with bacteria-loaded moisture that enters through stomates. Bacteria can infect mature tissue if there are wounds (Timmer 1991). Necrotic lesions develop in leaves, stems and fruit. Tree defoliation, blemished fruit, precocious fruit drop, twig dieback and tree decline are symptoms of severe infections (Schubert et al. 2001).

Canker B and C are strains of *X. axonopodis* pv. *aurantifolii*. The former was isolated from lemon, 'Mexican' lime, sour orange and pummelo in Argentina, Uruguay and Paraguay. The latter was isolated from 'Mexican' lime in Brazil. Different strain (A*) was also isolated from 'Mexican' lime grown in Saudi Arabia, Oman, Iran and India (Gottwald et al. 2002; Civerolo 1985).

Asiatic citrus canker severely affects grapefruit, some sweet oranges like 'Hamlin', 'Pineapple' and 'Navel', 'Mexican' ('Key') lime, lemons, and trifoliate orange and its hybrids (Gottwald et al. 2002). Bactericidal sprays and pruning at two-week intervals reduced canker incidence (Kapur et al. 1997). Copper sprays and other chemicals have also been effective (Zhihua et al. 2001; Kale et al. 1994). However, in moist subtropical and tropical climates chemical control is not commercially feasible (Gottwald et al. 2002; Schubert et al. 2001). Replacing susceptible varieties by field resistant ones seems to be a solution.

Selection or breeding for canker resistance is time-consuming but is a promising option. In fact, interesting results were reported for lemon and lemon hybrids artificially inoculated and evaluated under field conditions for canker resistance (Prasad et al. 1997). However, a reliable screening protocol is necessary for canker resistance, since several

factors influence the consistency of screening tests using artificial inoculations under controlled conditions.

Inoculation technique affects the infection response. In fact, pinprick inoculation induced a similar reaction in citrus and citrus hybrids, showing general susceptibility of leaf mesophyll. Wounds do not simulate the natural infection process, and bypass stomatal infection allowing the mesophyll colonization by bacteria (Gottwald et al. 1993). Moreover, 'Tahiti' lime and 'Mexican' lime showed similar susceptibility in experiments performed in greenhouse conditions, while field experiments confirmed that 'Tahiti' lime is more tolerant. Spray inoculation was used in both cases in greenhouse and field experiments (Leite and Mohan 1984). Greenhouse temperature and spray inoculation frequency may have affected the results.

The leaf physiological stage also plays an important role in the development of infection reaction. Leaves are highly susceptible to stomatal infections at two-thirds to full expansion stage (Graham et al. 1992). As leaves age, mesophyll resistance increases affecting bacterial growth (Stall et al. 1982). 'Lakeland' limequat was chosen as a breeding progenitor owing to the known potential for Asiatic canker resistance, as a hybrid of *Fortunella japonica*. It was identified as highly resistant to Asiatic citrus canker after field evaluation (Reddy 1997).

CHAPTER 2 IMPROVEMENT OF ACID CITRUS FRUIT CULTIVARS VIA INTERPLOID HYBRIDIZATION

Introduction

Lemon (*C. limon* (L.)Burm. f.) is the most important acid citrus fruit in terms of commercial significance. It was identified as a hybrid of sour orange (*Citrus aurantium*) and citron (*Citrus medica* L.) through chloroplast and nuclear genome analysis (Gulsen and Roose 2001). In general, lemon fruit are variable in shape; spherical in certain varieties and prolate spheroid in most commercial varieties, with a mamilla or nipple at the stylar end, and a variable number of seeds per fruit. *Citrus aurantifolia* Swing. is considered a true acid lime, with small, spherical and seedy fruit. The most common variety is 'Mexican' lime (also known as 'West Indian' or 'Key' lime). Molecular analysis suggested that *C. medica* and *Citrus micrantha* Wester. were its progenitors (Nicolosi et al. 2000). *Citrus latifolia* Tan. is also an acid lime, with larger spherical seedless fruit. It is a triploid hybrid of unknown origin. 'Tahiti' or 'Persian' lime is the most commercially grown variety. 'Bearss' was initially considered a variety of 'Tahiti' lime, but it did not differ sufficiently from 'Tahiti' lime to be considered a new variety.

Acid citrus fruits have multiple uses including beverage flavoring, cattlefeed, culinary, industrial and medicinal applications (Swisher et al. 1977; Morton 1987). Both lemon and lime require different climatic conditions for optimal production. Lemon is more adapted to sub-tropical conditions with low-humidity atmosphere; even so most of

the growing lemon areas are periodically exposed to freezes. Limes are tropical species that are generally grown in warm and humid areas of the subtropics.

The worldwide acid citrus fruit industry requires new varieties not only to satisfy a fresh market that demands high quality seedless fruit with high acid content and the manufacturing industry that calls for high quality juice and oil, but also to develop other horticultural traits to minimize the more threatening citrus diseases such as Asiatic citrus canker, 'mal secco' fungus, citrus tristeza virus and witches' broom disease of lime.

Furthermore, since lemon and lime are cold sensitive, improving cold hardiness is another important objective. In Florida, in addition to the unsuitable climatic conditions necessary to produce a high quality lemon, the Great Freeze of 1894-1895 caused a shift in the purpose for lemon production. Frozen lemon concentrate became the primary product. Searching for new varieties, two hundred lemon selections were planted in Florida in 1950 to evaluate their horticultural performance, considering yield of acid on a per acre basis and high grade of peel oil as the main targets. A few clones were selected as promising for processing (Knorr 1958).

'Bearss' lemon and the hybrid 'Meyer' lemon (*Citrus meyeri* Tan.) are the current acid citrus fruit varieties that make up the small Florida lemon industry with a total of 1000 bearing acres in 2000 (Florida Agricultural Services 2000). 'Mexican' and 'Tahiti' lime production in south Florida has been devastated by the eradication program to control a severe bacterial disease, Asiatic citrus canker (*Xanthomonas axonopodis* pv. *citri*). The eradication program is still underway; with 3,168 acres destroyed in 1996, and only 388 acres of limes remaining at the end of 2002 (Florida Agricultural Services, 2002).

In California, 'Eureka' x 'Lisbon' crosses did not succeed. Inbreeding depression is characteristic of citrus hybrids from narrow crosses, while hybrid vigor is present in progenies from wider crosses (Soost and Roose 1996). Under these circumstances, interploid hybridization becomes an interesting approach, considering that the fresh citrus market desires seedless fruit. Also, the availability of new promising allotetraploid somatic hybrid acid fruit breeding parents has made this approach more attractive. A somatic hybrid germplasm pool that combines together diverse genome combinations from citrus and citrus relatives is now available (Grosser et al. 1998; 2000).

Interploid crosses, either diploid x tetraploid or the reciprocal, are being increasingly used in citrus to generate seedless hybrids (Cameron and Burnett 1978; Starrantino 1992; Tusa et al. 1996; Cameron and Soost 1969). Triploid recovery is to a large extent precluded by precocious endosperm development and polyembryony. Endosperm degeneration was evident 7-10 weeks after fertilization of monoembryonic seed parents in diploid x tetraploid crosses; in consequence, a high number of embryos failed to develop, reducing the frequency of viable triploid hybrids. Higher proportions of triploid hybrids were generated from tetraploid x diploid crosses using monoembryonic seed progenitors (Cameron and Burnett 1978).

The 3:5 ploidy ratio of embryo to endosperm also induced seed abortion in crosses using ^{males?} 4X females, but embryo and endosperm developed normally, and seeds were small (Esen and Soost 1973). Similarly, 5X endosperm of polyembryonic seeds showed precocious development, regardless of the presence of nucellar embryos (Wakana et al. 1981). It was hypothesized that the unbalance of chromosome numbers between embryo and endosperm disturbs the relationship between them causing endosperm abortion and

subsequently embryo failure (Esen and Soost 1973). According to the endosperm balance number hypothesis, the 2:1 maternal:paternal ratio in the hybrid endosperm itself determines the normal endosperm development (Carputo et al. 1999). This hypothesis explains the differences obtained in diploid x tetraploid crosses and their reciprocal.

Embryos are 3X in both cases but the maternal:paternal ratios in the endosperm are $2x \times 4x$ 1:1 $4x \times 2x$ and 4:1 in diploid x tetraploid and tetraploid x diploid crosses respectively (Johnston et al. 1980).

To increase triploid hybrid recovery, monoembryonic seed parents are recommended, since occurrence of nucellar embryos affects survival and normal development of zygotic embryos (Soares Filho et al. 1992). However, polyembryony is a trait present in most citrus species, including acid citrus fruit varieties. Nonetheless, the efficiency of hybrid recovery has improved owing to the application of the embryo culture technique. This study was performed to generate triploid progenies of acid citrus fruit through interploidy hybridization. The ultimate goal is to produce acid lime and lemon-type fruit with potential for seedlessness, cold tolerance and disease resistance. Several variables related to pollination and fertilization efficiency are discussed.

Materials and Methods

Plant Material

Diploid x tetraploid crosses and some reciprocals were carried out during three successive years. During the 2000 season, the female breeding progenitors were the following diploids: 'Key' lime (*Citrus aurantifolia* (Christm.) Swing.), 'Lakeland' limequat hybrid (*Citrus aurantifolia* x *Fortunella japonica*), 'Palestine sweet lime' (*Citrus limettoides* Tan.), 'Etrog' citron (*Citrus medica* L.) and two lemon (*Citrus limon* (L.) Burm. f.) varieties; 'Lisbon' and 'Todo el Año'. Two allotetraploid somatic hybrids

'Hamlin' orange + 'Femminello' lemon (HF) and 'Valencia' orange + Rough lemon (VRL) were also included as female parents. The pollen progenitors were the following three somatic hybrids: HF, 'Key' lime + 'Valencia' orange (KLV) and Milam hybrid + 'Femminello' lemon (MF); and two autotetraploids 'Giant Key' lime (GKL) and 4X 'Femminello' (4XF). The diploids 'Lakeland' limequat and 'Key' lime were also used as pollen parents.

In the 2001 season, five lemon cultivars grown at the University of Arizona were added to the previous group of seed progenitors as follows: 'Limoneria 8A', 'Limoneiro fino 49', 'Femminello Santa Teresa', 'Arrancino' and 'Genova'. Additionally, 'Valencia' orange + 'Femminello' (VF) and VRL somatic hybrids were utilized as seed and pollen progenitors. During the 2002 season, two interploid crosses were performed: 'Lisbon' x HF and 'Todo el Año' x HF. The Florida Citrus Arboretum-Division of Plant Industry (Winter Haven, FL), Citrus Research and Education-IFAS-University of Florida, and Orie Lee Groves (St. Cloud, FL) were the locations where most of the breeding progenitors were grown, unless otherwise stated.

Pollination

Flower buds from pollen parents were collected when they were close to anthesis. Excised anthers were placed into Petri plates to be dehydrated over silica gel overnight. Dry pollen was kept at 4°C until use. Viability of dry pollen grains was determined using acetocarmine staining (1%). Viable (red) and non-viable (yellow) pollen grains (Fig. 2.1) were counted on five microscope fields (200X) from two slide samples per pollen progenitor.

Alternatively, flower buds from female parents were selected considering the proximity to anthesis and bud size in order to maximize uniformity of the physiological

age. After petal removal and emasculation, a portion of pollen was placed on the stigma. Flowers were tagged indicating pollen source, number of flowers and date. Harvest dates varied according to seed progenitors, about 60-90 days for 'Key' lime and 'Lakeland' limequat, and 90-110 days for the other progenitors.

Seed and Embryo Extraction

Fruit were surface-sterilized by immersion in a solution of 20% commercial bleach (5.25% NaOCl) and 2-3 drops/ liter Tween 20 for at least 30 min. Under aseptic conditions, dry fruits were cut at the equatorial zone avoiding the core where seeds are embedded. Both halves were twisted in opposite direction until total separation. Seeds were grouped according to size; small= small seed without endosperm remaining; medium= intermediate seed size with semi-solid or degenerated endosperm, and large= fully developed seed.

Embryos were carefully excised and categorized into three groups: globular, heart and early cotyledonary embryo; immature cotyledonary embryo; and mature embryo. Embryo excision was performed under 100X magnification using a stereomicroscope. Excised embryos were immediately plated on nutrient media to induce germination. Nutrient media and culture conditions are detailed in chapter 3. Fruit set, seed number/fruit, empty seed number, and number of embryos per developmental stage were recorded and analyzed in each cross combination.

Ploidy Analysis

After culturing seedlings on growth medium for a month, most of the plants were ready to be transferred to soil. Before soil transplant, ploidy level was determined by flow cytometry. A small piece of leaf sample and diploid standard were chopped simultaneously in extraction buffer, after filtration with 20- μ m mesh; the nuclei were

stained with fluorescent dye. Extract buffer and staining solution were obtained from a Partec high resolution staining kit. The relative amount of nuclear DNA content (C-value) was measured using a ploidy analyzer (Partec GMBH, Münster-Germany). Polyploids were planted in 38-well trays containing a commercial soil mixture of Metro Mix 500 (The Scotts Co., Marysville, OH). Plants were covered with a plastic lid for two weeks and kept under partial shade for acclimatization. After two weeks, seedlings were fertilized with 2-3 g/L Peters (Division of United Industries Corp. St. Louis, MO), which is a soluble fertilizer (20-20-20 with micronutrients). Minerals elements were periodically supplied to old seedlings using Essential Minor Elements (Agricultural Insecticides, Inc., Palmetto, FL) and Osmocote (The Scotts Co., Marysville OH).

Results

Viability of dry pollen, estimated by acetocarmine staining, was high for most of the progenitors except for 'Key' lime and KLV in the 2000 season (Table 2.1). A similar trend was observed in 2001, although viability was higher in all the pollen sources. Pollen of KLV even in fresh condition showed lower viability (59.93%). After the cold weather period, 'Key' lime and the 'Lakeland' limequat hybrid flowered continuously, with occasionally heavy blooming. In 2000, flowering began in February, while a two-month delay was evident in 2001. The other female progenitors showed a flowering peak in March.

Fruit set of individual female progenitors was evidently affected by the pollen progenitors. In diploid x tetraploid crosses accomplished in the 2000 (Table 2.2), 'Key' lime as a seed progenitor experienced the lowest fruit set regardless of the pollen progenitor, while lemons and 'Etrog' set high percentages of fruit. Regarding the pollen progenitors, HF resulted in high percentages of fruit set on lemons, 'Lakeland' limequat

and 'Palestine sweet' lime. In spite of the low pollen viability of KLV, acceptable fruit set was recorded from all female parents with the exception of 'Key' lime and 'Lakeland' limequat. Relating to seed number per fruit, only those highly seedy cultivars, such as 'Lisbon' and 'Etrog' showed noticeable variation of seed number depending upon the pollen progenitor. A high proportion of large seeds were observed in 'Lakeland' limequat and 'Lisbon' lemon and some crosses with 'Palestine' sweet lime. Quantity of empty seed was affected by pollen progenitor on crosses made on Lisbon, Etrog and Palestine sweet lime. The HF pollen showed a tendency to induce more seeds, with a notably large proportion of empty seed in 'Lisbon'.

Triploids were identified in all progenies from diploid x tetraploid cross of the 2000 season, when ploidy analysis was applied to normal seedlings (Table 2.3). Generally, a higher proportion of triploids developed from immature embryos, however, an exception was the triploids from mature cotyledonary embryos of 'Etrog' x KLV. Total number of triploids was from 'Key' lime 34, 'Etrog' 28, 'Lisbon' 32 and 'Todo el Año' 25. Considering the triploid population regarding the total seed number per parent, the percentages were as follows: 8.92, 8.69, 6.03 and 10.25, respectively.

Small numbers of triploids developed from 'Lakeland' limequat (9) and 'Palestine sweet' lime (13), which corresponded to 3.25% and 6.81% triploids, respectively. Analyzing the fertility of pollen parents, the triploid frequencies were similar for HF (6.70), KLV (6.81), and 4X Femminello (7.14) and very low for GKL (1.83). A few tetraploids were obtained from 'Key' lime, 'Lakeland' limequat and 'Lisbon' lemon. Higher levels of ploidy were observed when the seed parent was tetraploid. A pentaploid

was generated from HF x 'Lakeland' limequat and a hexaploid from HF x 'Palestine sweet' lime.

The repeated cross combinations in the 2001 season (Tables 2.4 and 2.5) showed a similar tendency for the previously discussed parameters. The fruit set of 'Key' lime showed a general improvement, and the proportion of large seed was reduced not only in 'Key' lime but also in 'Lakeland' limequat. On the contrary, 'Palestine sweet' lime produced a considerably lower fruit set; but this inconvenience was associated with drought stress encountered by the non-irrigated seed trees used. An increment of KLV pollen viability was related with considerably higher fruit set and seed number per fruit in lemons (Lisbon and Todo el Año). Pollen of VF induced high fruit set in 'Lisbon' and 'Todo el Año', however about half of the total seed (53%) were empty.

The five lemon cultivars grown in Arizona performed differently. 'Limonero fino 49' is a very seedy cultivar that showed the highest fertility in all crosses, excluding the cross with VF (Table 2.4). 'Limoneria 8A', 'Santa Teresa Femminello' and 'Genova' did not set fruit when the pollen progenitor was either VF or KLV. Unlike the others, 'Arancino' set some fruit from three different pollen parents; however all of them lacked seeds. In general, most of the lemon parents developed high numbers of empty seeds, which could reach about 50 % of the total, or even higher in the case of 'Limonero fino 49' (60-69%). 'Genova', 'Todo el Año' x HF. 'Todo el Año' x KLV showed lower frequencies of empty seed.

The number of triploids generated in 2001 was noticeably high in all seed progenitors except 'Key' lime (12), 'Palestine sweet' lime (6) and 'Etrog' (5) (Table 2.6). The frequency of triploids based on seed number was 5.52%, 24.0% and 1.13%

respectively. 'Lakeland' limequat produced an increased amount of triploids (16), but doubled the percentage of recovered hybrids (7.92%). All lemon fertile crosses developed high numbers of triploids per seed parent as follows; 34 'Lisbon', 172 'Todo el Año', 18 'Limoneria 8A', 69 'Limonero Fino 49' and 14 'Femminello Santa Teresa' (Table 2.7). The corresponding triploid proportions were 9.47%, 20.38%, 7.89%, 7.57% and 16.87%, respectively. An exception was the 'Genova' x VRL cross that generated only one hybrid.

The frequency of triploid production based on the pollen parent was also higher in the second season; 14.41%, 16.35%, 9.55% and 9.58% for HF, KLV, VF and VRL, respectively in lemon crosses. Lower percentages for HF (3.91%) and VF (2.73%) were observed in the other acid citrus seed parents, while KLV was similar (15.42%). A high frequency of triploid lemon hybrids developed from mature cotyledonary embryos. Unlike the first season, a higher frequency of tetraploids was obtained from fully developed seeds of 'Todo el Año'. Similarly, the other lemon parents gave rise to tetraploids except 'Genova'.

In tetraploid x diploid crosses, HF and VF as seed progenitors brought about very different results in relation to their reciprocals. It was only possible to harvest a few fruit from HF, with small seed numbers (Tables 2.8 and 2.9). As in diploid x tetraploid crosses, fruit set, seed per fruit and empty seed were determined in large extent by the pollen parents. Although fruit set increased for 'Key' lime and 'Lakeland' limequat in the second season, triploids were only obtained in the first season.

All fruit prematurely fell off the tree from cross-pollinated VF trees; however when VF was the pollen parent, hybrids were recovered from four out eleven cross

combinations. Likewise, VRL was very incompetent as a seed progenitor, since no fruit was obtained from three different crosses carried out on five different plants grown in two locations in the 2000 and 2001 seasons. On the contrary, VRL as a pollen parent produced fertile crosses with 'Limoneria 8A', 'Limonero Fino 49' and in less extent with 'Genova', excluding 'Arancino' and 'Femminello Santa Teresa'.

In 2002, 'Todo el Año' x HF did not show a remarkable difference in fruit set, but the number of seed per fruit was considerably larger than the two previous seasons. 'Lisbon' lemon had better fruit set compared to the first season, and a similar number of seed per fruit (Table 2.10). The proportion of empty seed was also high (51- 59%). The number of triploids transferred to soil was 110 and 66 for 'Todo el Año' and 'Lisbon', respectively.

Discussion

Embryo culture technique allowed the recovery of numerous triploid progenies of acid citrus fruit resulting from interploid hybridization. In spite of the polyembryonic character of the female diploid progenitors, hybrids were recovered from all except 'Arancino' lemon. In citrus interploid hybridization, sexual tetraploids are recommended as seed parents instead of partly apomictic tetraploids and diploids, since a high proportion of triploids can be generated (Cameron and Burnett 1978).

In this study, very few hybrids were obtained from polyembryonic seed progenitors in tetraploid x diploid crosses, HF is highly polyembryonic; a maximum of 10 embryos per seed was recorded. However, the major limitation was the inferior fruit set in all allotetraploid somatic hybrids. It is likely that factors related with fertilization and ploidy ratio affected fruit set of controlled pollinations, since fruit developed from open pollinated flowers. Lower fruit set and seeds were reported for autotetraploid mandarins

in tetraploid x diploid crosses compared to their reciprocals (Khan et al. 1996). Generally allotetraploid somatic hybrids are used as citrus pollen progenitors (Tusa et al. 1996; Deng et al. 1996; Oiyama et al. 1991).

Zygotic embryos are more likely to be present in small seeds; however, a high proportion of small-germinated embryos did not develop into normal plants. Perhaps the tested media did not meet the embryo requirements for complete development, considering that embryo development is dynamic system that continually changes during maturation (Ramming 1990). Another factor that cannot be disregarded is the presence of aneuploids in the new hybrids. Esen and Soost (1972a) determined that most frequent viable aneuploids were $3X + 1 = 28$ (24.80%) and $4X + 1 = 37$ (45.90%) in a seedling population from diploid x tetraploid crosses. They found a wide range of aneuploid chromosome numbers in developing embryos (22-41 chromosomes). The number of extra or absent chromosomes, as well as the type of information stored in the extra or missing chromosomes may affect the plant viability and vigor. Aneuploidy could have also been present in rescued embryos with very low vigor.

'Key' lime and 'Lakeland' limequat as seed progenitors showed low fertility; many fruit dropped off the trees during the first two weeks after pollination and small frequencies of hybrids were recovered from different crosses. These progenitors also had a low number of viable seed per fruit, with 2-3 embryos per seed. In the second season, more immature embryos were obtained from both seed parents by reducing the harvest time to 60 days after pollination instead of three months. Unexpectedly, some 'Key'-lime fruit lacked seeds or contained a few in the second season. 'Palestine sweet' lime showed low fertility in the first season, but higher triploid frequency was recorded from

the viable crosses in 2001. In open-pollinated seedlings, sampling year and cultivar influenced the frequency of zygotic vs. nucellar embryos. Crop load, plant age, and nutrient status might also affect the frequency of zygotic embryos (Khan and Roose 1988).

'Etrog' citron, in spite of being a very seedy cultivar (approximately 117 seeds/ open-pollinated fruit), produced a percentage of triploid recovery ranging between 11 and 25%, with most of them developing from mature embryos. The incompatibility of 'Etrog' x VF was evident owing to the drastic reduction of seed/ fruit and the lack of hybrids recovered.

Lemons also developed considerably large numbers of seeds. Despite the high proportion of empty seeds in lemons, particularly 'Limonero Fino 49' (60-69%) and 'Limonera 8A' (53%), high numbers of viable seeds were obtained. The less seedy lemons ('Genova' and 'Femminello Santa Teresa') had a low proportion of empty seeds. In lemons, most of the small empty seeds were indeed ovules whose embryos aborted at a very early stage. Ovules were very small without any visible remnants of embryo or endosperm. Esen (1971) determined that partial and fully developed seeds arise from fertilized ovules.

The VF somatic hybrid showed the lowest sexual compatibility with most of the seed parents. Despite its high pollen viability (Table 2.1), hybrids were only recovered from crosses with 'Key' lime, 'Lakeland' limequat, and the 'Todo el Año' and 'Lisbon' lemons; the other seed parents did not set fruit at all, except 'Arancino', but the fruit were seedless. Fruit were collected from 'Etrog' x VF, however they contained lower number

of viable seeds and large amount of empty seed compared to the other crosses made on 'Etrog' citron.

The high frequency of tetraploid seedlings obtained from fully developed seeds was rather consistent in lemon progenitors, with a very low incidence in 'Key' lime and 'Lakeland' limequat, and totally absent in the other seed progenitors. Similarly, Tusa et al. (1996) reported a high proportion of tetraploids in progenies from diploid x tetraploid crosses using the polyembryonic 'Femminello' lemon as female parent. Studies on seed development of monoembryonic citrus from diploid x tetraploid crosses demonstrated that seeds with a tetraploid embryo and hexaploid endosperm develop normally. A remarkable difference was observed between the developmental stages of triploids and tetraploids; tetraploid embryos were larger and more advanced in development in relation to triploids (Essen and Soost 1973), which is comparable to results reported for lemon triploid and tetraploid embryos. It was proven that tetraploid seedlings originated from fertilization of unreduced gametes of monoembryonic diploid female parents (Essen and Soost 1972b).

In monoembryonic seed progenitors, the hybrids were often tetraploid in diploid x tetraploid crosses (Cameron and Soost 1969). Spontaneous tetraploids developing from nucellar tissue have also been reported in citrus (Soost and Roose 1996). Tetraploid hybrids and spontaneous autotetraploids were also identified in mandarin progenies obtained from polyembryonic seed parents in diploid x tetraploid crosses (Kaneyoshi et al. 1997). In any case, it is necessary to confirm the origin of those tetraploids to judge their significance. Tetraploid hybrid progenies would increase the genetic variability and

availability of breeding parents for interploid hybridization. So far, nucellar origin was established for 'Lakeland' limequat tetraploids via RAPD analysis.

In this study, a genetically diverse population of acid citrus fruit triploids has been generated to provide a pool of genetic variability for further horticultural evaluation and selection of seedless varieties. The unique genomic composition of the allotetraploid somatic hybrids and degree of compatibility with the acid citrus fruit cultivars selected, confirmed their potential as breeding pollen progenitors. Furthermore, the genomes of the selected somatic hybrids contain half of either lemon or lime genetic composition; therefore they have potential to produce new seedless acid citrus fruit progenies with more cold hardiness and disease resistance (See chapter 5; Tusa et al. 2000).

Moreover, progenies from interploid hybridization also possess a high potential for improved oil quality and content in correspondence with previous reports for lemon triploid hybrids (Gazea et al. 1996; Ruberto et al. 1997) and the allotetraploid somatic hybrid 'Valencia' orange + 'Femminello' lemon (Fatta del Bosco et al. 1998). It was also established that fruit of triploid lemons had high acid percentages; however this variable was affected by cross direction (Soost and Cameron 1969). Ornamental use is an underexploited use of citrus trees. Citrus and citrus relatives are suitable for landscaping and different ornamental uses according to an Italian study about the potential use of citrus as ornamentals (Continella et al. 1992). New progenies may also have value as dooryard trees or ornamentals.

Table 2.1. Pollen viability according to acetocarmine staining assay of staminate progenitors utilized in the crosses of the 2000 and 2001 seasons.

Pollen Progenitor	Viable Pollen Grains (%)	
	2000 Season	2001 Season
KL V	25.27	69.65
HF	76.32	80.54
VRL	-----	80.22
VF	-----	85.15
MF	80.61	-----
GKL	55.80	76.74
4XF	88.89	90.93
Lakeland limequat	85.87	90.71
Key lime	7.73	46.26
Palestine sweet lime	87.60	-----

KL V: 'Key' lime + 'Valencia' orange; HF: 'Hamlin' orange + 'Femminello' lemon;
 VRL: 'Valencia' orange + 'Rough lemon'; VF: 'Valencia' sweet orange + 'Femminello'
 lemon; MF: 'Milam' hybrid + 'Femminello' lemon; GKL: 'Giant Key' lime; 4XF:
 Autotetraploid 'Femminello'.

Table 2.2. Fruit set and seed number of acid citrus progenies obtained from diploid x tetraploid crosses completed in the 2000 season.

Progenitors			Seed									
Seed	Pollen	Flower	Fruit	Fruit Set (%)	Total	Seed/ Fruit	Small		Medium		Large	
							Embryo	Empty	Embryo	Empty	Embryo	Empty
Key lime	HF	300	25	8.33	150	6.00	20	15	77	9	29	
	4XF	20	1	5.00	4	4.00	0	0	4	0	0	
	KLV	381	42	11.02	227	5.40	22	25	123	9	48	
Lakeland limequat	HF	47	14	29.79	84	6.00	2	8	23	8	43	
	GKL	66	16	24.24	109	6.81	1	3	26	8	71	
	KLV	137	16	11.68	127	7.94	9	21	33	8	56	
Palestine sweet lime	4XF	93	14	15.05	80	5.71	1	5	42	22	10	
	HF	17	8	47.06	40	5.00	11	7	18	2	2	
	MF	22	4	18.18	36	9.00	6	4	12	2	12	
Etrog citron	KLV	21	7	33.33	56	8.00	15	0	10	3	28	
	HF	12	3	25.00	189	63.00	15	32	54	19	69	
	KLV	9	3	33.33	133	44.33	3	0	21	10	99	
Lisbon lemon	HF	21	9	42.86	375	41.67	47	172	83	22	51	
	KLV	23	6	26.09	156	26.00	21	22	32	7	75	
Todo el Año lemon	HF	11	9	81.82	147	16.33	4	56	40	16	31	
	KLV	20	6	30.00	97	16.17	15	26	26	17	13	

KL.V: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon'; 4XF: 'Valencia' orange + 'Femminello' lemon; MF: 'Milan' hybrid + 'Femminello' lemon; GKL: 'Giant Key' lime; 4XF: Autotetraploid 'Femminello'.

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + Rough lemon;

VF: 'Valencia' orange + 'Femminello' lemon; MF: 'Milam' hybrid + 'Femminello' lemon; GKL: 'Giant Key' lime; 4XF: Autotetraploid 'Femminello'

Table 2.3. Plant recovery and ploidy level of acid citrus progenies generated from diploid x tetraploid crosses performed in the 2000 season.

Progenitors		Embryo Developmental Stage											
		Globular-Early Cotyledonary						Large Immature Cotyledonary					
		Seed	Pollen	%G	%NP	2X	3X	4X	%G	%NP	2X	3X	4X
Key lime	HF		HF	75.51	46.15	36	6	0	100	60.20	49	10	0
	4XF		4XF	0	0	0	0	0	80.00	40.00	2	2	0
	KLV		KLV	54.90	42.62	21	5	0	100	70.20	103	2	1
Lakeland limequat	HF		HF	35	55.56	3	2	0	0	0	0	0	0
	GKL		GKL	66.67	28.57	2	0	0	0	0	0	0	0
	KLV		KLV	36.00	28.57	2	2	0	100	50.00	9	0	0
Palestine sweet lime	4XF		4XF	37.04	28.57	0	4	0	100	0	0	0	0
	HF		HF	85.00	48.78	16	4	0	100	50.00	2	0	0
	MF		MF	62.50	62.5	2	3	0	100	71.43	15	0	0
Etrog citron	KLV		KLV	100	23.53	7	1	0	100	100	9	0	0
	HF		HF	42.86	60.00	1	8	0	0	0	0	0	0
	KLV		KLV	75.00	8.70	0	2	0	0	0	0	0	0
Lisbon lemon	HF		HF	43.52	64.81	16	19	0	60.00	50.00	3	1	0
	KLV		KLV	45.10	41.94	7	6	0	0	0	0	0	0
	HF		HF	65.38	38.89	1	6	0	94.44	65.38	9	8	0
Todo el Año lemon	KLV		KLV	18.52	16.67	1	0	0	86.67	50.00	2	6	0
	HF		HF	65.38	38.89	1	6	0	94.44	65.38	9	8	0
	KLV		KLV	18.52	16.67	1	0	0	86.67	50.00	2	6	0

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon';
 VF: 'Valencia' sweet orange + 'Femminello' lemon; MF: 'Milam' hybrid + 'Femminello' lemon; GKL: 'Giant Key' lime; 4XF: Autotetraploid 'Femminello'; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing plant; 2X, 3X, 4X: Ploidy level.

Table 2.4. Fruit set, seed and embryo number of acid citrus progenies from diploid x tetraploid crosses completed in the 2001 season.

	Progenitors		Flower	Fruit	Fruit Set (%)	Total	Seed/ fruit	Seed									
	Seed	Pollen								Small		Medium		Large			
										Embryo	Empty	Embryo	Empty	Embryo	Empty		
Key lime	HF	102	20	19.61	54	2.70	1	8	31	12	2						
	VF	106	25	23.58	98	3.92	9	14	40	33	2						
	KLV	111	16	14.41	65	4.06	7	16	28	14	0						
Lakeland limequat	HF	82	21	25.61	106	5.05	4	14	74	12	2						
	VF	87	11	12.64	58	5.27	3	9	22	15	9						
	KLV	71	6	8.45	38	6.33	2	0	23	5	8						
Palestine sweet lime	HF	27	2	7.41	5	2.50	1	1	2	1	0						
	VF	26	0	0	0	0	0	0	0	0	0						
	KLV	11	3	27.27	20	6.67	9	2	8	1	0						
Etrog citron	HF	5	4	80.00	270	67.5	12	17	27	17	197						
	VF	9	6	66.67	174	29.0	2	53	76	43	0						

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon.

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon.

Table 2.5. Lemon fruit set, seed and embryo number obtained from diploid x tetraploid crosses completed in the 2001 season.

Progenitors			Flower	Fruit	Fruit Set (%)	Total	Seed					
Lemon Seed	Pollen						Small		Medium		Large	
							Embryo	Empty	Embryo	Empty	Embryo	Empty
Todo el Año	HF	27	20	74.07	406	20.3	13	55	164	99	75	
	VF	16	10	62.50	157	15.7	8	36	56	47	10	
	KLV	29	15	51.72	281	18.73	11	19	118	39	94	
Lisbon	VF	9	7	77.77	199	28.43	3	70	40	35	51	
	KLV	8	3	37.50	160	53.33	15	78	52	6	9	
Limoneria 8A	HF	15	8	53.33	125	15.63	0	33	44	32	16	
	VF	15	0	0	0	0	0	0	0	0	0	
	KLV	15	0	0	0	0	0	0	0	0	0	
Limonero	VRL	19	6	31.58	103	17.17	0	19	31	37	16	
	HF	15	12	80.00	503	41.92	34	205	142	96	26	
	VF	20	0	0	0	0	0	0	0	0	0	
Fino 49	KLV	15	7	46.67	140	20.00	2	45	42	49	2	
	VRL	15	7	46.67	268	38.29	15	94	55	92	12	
Feminello Santa Teresa	HF	15	5	33.33	83	16.60	0	8	47	18	10	
	VF	15	0	0	0	0	0	0	0	0	0	
	KLV	15	0	0	0	0	0	0	0	0	0	
Arancino	HF	16	4	25	0	0	0	0	0	0	0	
	VF	15	1	6.67	0	0	0	0	0	0	0	
	KLV	15	1	6.67	0	0	0	0	0	0	0	
Genova	VRL	17	0	0	0	0	0	0	0	0	0	
	HF	15	0	0	0	0	0	0	0	0	0	
	VF	15	0	0	0	0	0	0	0	0	0	
	KLV	15	0	0	0	0	0	0	0	0	0	
	VRL	15	1	6.67	22	22	3	3	13	0	3	

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Feminello' lemon; VF: 'Valencia' sweet orange + 'Feminello' lemon; VR: 'Valencia' sweet orange + Rough lemon.

Table 2.6. Plant recovery and ploidy level of acid citrus progenies derived from diploid x tetraploid crosses completed in 2001.

Progenitors		Embryo Developmental Stage																
		Globular- Early Cotyledonary						Large Immature Cotyledonary						Mature Cotyledonary				
		Seed	Pollen	%G	%NP	2X	3X	4X	%G	%NP	2X	3X	4X	%G	%NP	2X	3X	4X
Key lime	HF	61.11	43.75	7	0	0	0	100	58.06	17	1	0	100	100	4	0	0	0
	VF	81.18	100.	13	4	0	100	93.55	26	2	1	100	100	10	0	0	0	0
	KLV	60.87	38.46	8	2	0	83.33	43.24	12	3	0	100	100	3	0	0	0	0
Lakeland limequat	HF	75.00	67.31	31	4	0	97.67	90.80	78	1	0	0	0	0	0	0	0	0
	VF	94.44	45.16	23	3	1	100	95	19	0	0	100	88.89	31	0	1	0	1
	KLV	65.00	36.67	7	4	0	88.00	76.47	7	4	0	100	100	8	0	0	0	0
Etrog citron	HF	77.27	31.58	2	5	0	0	0	0	0	0	0	96.59	93.26	77	6	0	0
	VF	100	62.5	10	0	0	95.16	93.22	59	0	0	0	0	0	0	0	0	0
Palestine sweet Lime	HF	66.67	40.00	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KLV	83.33	87.5	16	5	0	100	90.91	9	1	0	0	0	0	0	0	0	0

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon'; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing plant; 2X, 3X, 4X: Ploidy level.

Table 2.8. Fruit set, seed number and ploidy of rescued embryos from tetraploid x diploid crosses made in the 2000 season.

Progenitors		Flowers	Fruit	Fruit Set (%)	Seed			Large Embryo			
Female	Pollen				Total	Empty	Empty	%G	%NP	3X	4X
VRL	Key lime	70	0	0	0	0	0	0	0	0	0
	Lakeland limequat	79	0	0	0	0	0	0	0	0	0
	Palestine sweet lime	16	0	0	0	0	0	0	0	0	0
HF	Key lime	58	2	3.45	4	0	0	100	73.33	1	10
	Lakeland limequat	71	3	4.22	11	0	0	100	90.00	5	40
	Palestine sweet lime	5	1	20.00	7	0	0	100	60.53	0	23

VRL: 'Valencia' sweet orange + 'Rough lemon'; HF: 'Hamlin' sweet orange + 'Femminello' lemon; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing plant; 2X, 3X, 4X: Ploidy level.

Table 2.9. Fruit set, seed number and ploidy of rescued embryos obtained from tetraploid -x-diploid crosses completed in the 2001 season.

Progenitors	Seed	Pollen	Flower	Fruit	Fruit Set (%)	Seed/Fruit	Small Seed		Medium Seed		Globular-Early Embryo		Immature cotyledonary Embryo			
							Embryo	Empty	Embryo	Empty	%G	%NP	%G	%NP	3X	4X
HF	Key lime	48	5	10.42	4.2	3	9	0	9	0	92.31	100	100	87.50	0	14
VF	Lakeland limequat	38	3	7.89	2.33	1	1	1	4	1	100	90.91	0	100	0	4
VRL	Key lime	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lakeland limequat	42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon'; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing plant; 2X, 3X, 4X: Ploidy level.

Table 2.10. Fruit set, seed and embryo number of lemons from diploid x tetraploid crosses completed in the 2002 season.

Progenitors	Flower		Fruit		Seed/ fruit	Seed				Total
	Seed	Pollen	Number	Set (%)		Small		Medium		
						Embryo	Empty	Embryo	Empty	
Todo el Año	HF	24	21	87.50	887	76	483	206	36	86
Lisbon	HF	33	20	60.60	764	89	341	156	59	119
HF: 'Hamlin', orange + 'Femminello', lemon										

HF: 'Hamlin' orange + 'Feminello' lemon

Table 2.11. Plant recovery and ploidy level of lemon progenies generated from diploid x tetraploid crosses completed in 2002.

Progenitors		Embryo Developmental Stage																	
		Globular- Early Cotyledonary						Large Immature Cotyledonary						Mature Cotyledonary					
		Seed	Pollen	%G	%NP	2X	3X	4X	%G	%NP	2X	3X	4X	%G	%NP	2X	3X	4X	
Todo el Año		HF		75.11	72.14	54	45	0	64.86	45.24	6	78	14	98.82	96.47	42	43	13	
Lisbon		HF		68.02	73.75	71	46	0	69.23	30.45	9	42	0	100	100	59	23	8	
HF: 'Hamlin' sweet orange + 'Femminello' lemon; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing seedling; 2X, 3X, 4X: Ploidy level.																			

HF: 'Hamlin' sweet orange + 'Feminello' lemon; %G: Percentage Germination; %NP: Percentage of germinated embryos that developed into completely growing seedling; 2X, 3X, 4X: Ploidy level.

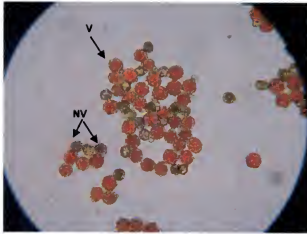


Figure 2.1 Pollen viability assay using acetocarmine staining. V: viable pollen grain; NV: non-viable pollen grains

CHAPTER 3

INDUCTION OF *IN VITRO* GERMINATION AND SEEDLING DEVELOPMENT OF IMMATURE EMBRYOS RESULTING FROM INTERPLOID HYBRIDIZATION IN ACID CITRUS FRUITS

Introduction

Lemon (*Citrus limon* (L.) Burm. f.), ‘Mexican’ or ‘Key’ lime (*Citrus aurantifolia* (Christm.) Swing.) and ‘Tahiti’ or ‘Persian’ lime *Citrus latifolia* Tan. are the principal acid fruit citrus fruit grown commercially worldwide. Lemon is mainly grown in the subtropical regions, while acid limes are restricted to the warmer climate of the tropics and sub-tropics. Freezes, diseases and pests continuously threaten the acid citrus fruit industry, causing considerable losses. New varieties that encompass resistance to adverse climatic conditions and maintain fruit quality are necessary.

Genetic improvement via sexual hybridization faces some constraints in citrus, such as polyembryony. In polyembryonic citrus, the zygotic embryo must compete for nutrients and space with embryos developed from nucellar tissue (Soost and Roose 1996). Soares-Filho et al. (1992) determined that sexual embryo size and survival were inversely related to the number of embryos per seed. Besides, identification of the sexual embryo might require extra analyses for identification, for instance cytology, flow cytometry, isoenzymes or molecular analysis (Tusa et al. 2002). The situation could be more complex in interploid crosses owing to post-zygotic incompatibilities that induce endosperm failure; in consequence, the number of viable offspring can be dramatically reduced.

In spite of the above-mentioned difficulties, interploid hybridization is still a very useful approach to generate new seedless progenies in citrus breeding programs. 'Oroblanco' (Soost and Cameron 1980) and 'Melogold' (Soost and Cameron 1985) are citrus triploid varieties that resulted from conventional breeding techniques. Interploid hybridization has become more efficient due to application of *in vitro* culture techniques that allow rescue of numerous undeveloped embryos. Success of embryo culture is to a large extent determined by the developmental stage of the embryo and the nutrients and plant growth regulators provided by the culture medium. Generally, young embryos (around 100 days after pollination) are excised from polyembryonic seeds (Deng et al. 1996; Tusa et al. 1996); in contrast seeds from monoembryonic seed parents were collected seven months after pollination or at maturity (Oiyama and Kobayashi 1990; Sykes and Lewis 1996).

Citrus embryo culture has been usually successful on two nutrient media: Murashige and Skoog (MS) (Murashige and Skoog 1962) and Murashige and Tucker medium (MT), which is a modified MS medium (Murashige and Tucker 1969). These two media have been adjusted to induce embryo germination. Mineral salts of MS (Starrantino 1992) or MS supplemented with malt extract and adenine sulfate induced a high germination rate of embryos obtained from monoembryonic seed parents (Starrantino and Reforgiato-Recupero 1981).

The addition of gibberellic acid (GA) to MS medium induced low germination rates of mandarin embryos (Khan et al. 1996). High germination rates (62-90%) were reported for mandarin embryos cultured on MS with high a sucrose concentration (6%) and GA (Sykes and Lewis 1996). The supplementation of MT with GA and malt extract

induced low (17.4-35.4%) germination rates of tangerine and pummelo embryos. Poorly germinated embryos required some addenda in the medium to develop new shoots (Deng et al. 1996). Undeveloped lemon and mandarin embryos were germinated on MT medium that had malt extract without plant growth regulators (Tusa et al. 1996), or with adenine (Oiyama and Kobayashi 1990).

Several factors were studied using polyembryonic diploid citrus. Pro-embryos required GA and a high sucrose concentration in MT, while early cotyledonary embryos germinated well on MS with malt extract. Germination media also affected plant recovery (Carimi et al. 1998). Genetic conformation and embryo developmental stage might influence the variable response of citrus aborted embryos to germinate on artificial nutrient media.

Most lemon and lime cultivars produce substantial numbers of embryos per seed (Soost and Roose 1996). Therefore, it is necessary to rescue embryos at an immature stage to reduce the risk of embryo failure and competition with nucellar embryos. The purpose of this study was to determine an appropriate culture media to induce germination of immature embryos and normal seedling development.

Materials and Methods

Plant Material

This study was performed utilizing immature embryos obtained from diploid x tetraploid crosses carried out during the 2001 and 2002 seasons, and a few reciprocals completed in 2001. Citrus diploid and tetraploid progenitors are specified in chapter 2, as well as the pollination procedure.

Embryo Excision

Fruit surface sterilization and the procedure for seed extraction and embryo excision are also provided in chapter 2.

Induction of Immature Embryo Germination

This experiment was performed with rescued embryos from crosses completed in the 2001 season. The germination treatments were combinations of two sucrose concentrations (146.06 and 219.11 mM) and two culture media, EME (Appendix A) and Gamborg's B5 basal medium (Gamborg et al. 1968). All media had 0.5 g/L malt extract, and 4.54 μ M GA. The pH of all media was adjusted to 5.8 using 1N KOH, prior to the addition of 7 g/L agar (Sigma Chemical Co., St. Louis, MO). GA was added to autoclaved media when the temperature was approximately 55°C. Immediately afterwards, 50 mL medium was poured into disposable Petri plates (100x20 mm).

To induce further seedling growth, germinated embryos were transferred approximately a month after germination onto MS medium. MS medium was supplemented with 20 g/L sucrose and 0.11 μ M 1-naphthalene acetic acid (NAA), and solidified with 8 g/L agar (Sigma Chemical Co., St. Louis, MO). Embryos and seedlings were grown under cool white fluorescent tubes with continuous light exposure at 28 °C.

Induction of Seedling Development

To achieve this study two crosses ('Lisbon' x HF and 'Todo el Año' x HF) were performed. Immature embryos rescued in globular, heart-shaped and early cotyledonary stages were germinated on Gamborg's B5 medium supplemented with 4.54 μ M GA, 0.5 g/L malt extract and 75 g/L sucrose. Germinated embryos were transferred onto 20 mL of growing media 15-17 days after of germination. The growing media were RMA

(Appendix B), MS and Gamborg's B5. All growing media contained 20 g/L sucrose, and RMA and MS contained 0.11 μ M NAA.

All mature embryos were germinated on MS basal medium with 20 g/L sucrose. The pH of all media was adjusted to 5.8 with 1N KOH prior to the agar addition. The agar (Sigma Chemical Co., St. Louis, MO) concentration was 7 and 8 g/L for germination and growth media, respectively. Germination was induced in disposable Petri dishes (100 x 20 mm) and plant growth in test tubes (20x100 mm). Four or five embryos were cultured per Petri plate to induce germination, and a single seedling was grown per test tube to induce plant growth. All *in vitro* cultures were grown at 28°C, under cool white fluorescent tubes with continuous light exposure.

Ploidy Testing

The procedures for ploidy level determination and soil transplant of recovered seedlings are specified in chapter 2.

Data Analysis

The medium and sucrose treatments were arranged in a 2x2 factorial. Data were subjected to one-way analysis of variance using SAS (Cary, NC). Four embryos were plated per Petri plate, with variable number of plates per treatment according to the number of available immature embryos, usually between 4-8 replicates. Percentage germination was measured per Petri plate a month after embryo plating, and percentage of normal plant (germinated embryos that developed into seedling with growing stem and root) was recorded a month after germinated embryos were grown on MS medium.

The induction of plant growth data was also subjected to one-way analysis of variance. In this experiment, five embryos were included per replicate and each replicate was repeated seven times. After four weeks, ploidy level, plant height, root length and

percentage of normal plant were evaluated. Fisher's least significant difference (LSD) or z test statistic procedure was applied to determine significance of differences among means.

Results

Induction of Immature Embryo Germination

Forty-one different cross combinations were made in the 2001 season. The number of immature embryos per combination varied greatly among lemon seed progenitors. 'Lisbon' and 'Todo el Año' developed the largest number of lemon embryos, whereas no lemon embryos were generated from 'Arancino' regardless of the pollen parent. Genova and 'Femminello Santa Teresa' generated embryos only when the pollen progenitors were HF and VRL (Table 3.1). The progenies from the other acid citrus fruit are summarized in the Table 3.2. 'Lakeland' limequat and 'Etrog' citron progenitors generated a high number of immature embryos. Nonetheless, most 'Etrog' embryos were in either advanced cotyledonary (3-7 mm) or fully developed stage. In those cases, embryos were grown on MS medium. Crosses including 'Key' lime and 'Palestine sweet' lime progenitors showed low fertility. Overall, the lowest number of embryos was generated from 4X x 2X crosses (Table 3.3).

The limited amount of immature embryos restricted the statistical analysis to the following crosses; 'Lakeland' limequat x HF, 'Todo el Año' x KLV, 'Todo el Año' x HF, 'Todo el Año' x VF, 'Limoneria 8A' x HF, 'Limonero Fino 49' x HF and 'Limonero Fino 49' x VRL. Germination of globular-early cotyledonary embryos significantly varied among cross combinations ($P=0.0155$) and between nutrient media ($P=0.0054$) (Table 3.4). Mean comparison by LSD procedure determined that the lowest germination (52.78%) of 'Limoneria 8A' x HF progenies was significantly different from all the other

embryos (Table 3.5). The medium B5 induced higher germination (82.89%) compared to EME medium (70.78%). Sucrose concentration in the media did not affect small embryo germination, however more embryos germinated at 204.50 mM (80.46%) compared with 146.07 mM (73.22%). The amount of small embryos that developed into normal plants varied significantly ($P=0.0269$) among cross combinations (Table 3.6). The lowest plant recovery was also obtained from 'Limoneria 8A' x HF (11.11%), and the highest from Lakeland limequat x HF (64.73%). No differences were determined among the other progenies (Table 3.5). Although germination medium did not affect significantly the amount of plant recovery, more normal plants were recovered when embryos germinated on B5 medium (41.51%) than on EME medium (35.02%).

Germination of immature cotyledonary embryos was studied on all previously mentioned crosses except 'Todo el Año' x VF and 'Limonero Fino 49' x VRL. Comparable to germination of small embryos, immature embryos also differed significantly ($P<0.0001$) among cross combinations. Germination was influenced by sucrose concentration ($P=0.0018$) and the cross combination-sucrose interaction ($P=0.0004$) (Table 3.7). Among cross combinations, 'Limonero Fino 49' x HF germinated at the lowest rate (65.30%) while 'Todo el Año' and 'Lakeland' limequat embryos had germination rates over 97 % (Table 3.8). Immature cotyledonary embryos germinated in larger percentage on media containing 204.50 mM sucrose (95.52%) in comparison with 146.07 mM (85.33%). Embryos of Limonero Fino 49 x HF were the most responsive to sucrose concentration, with germination varying from 45.50% to 65.07% when the media contained 146.07 and 204.50 mM, respectively (Table 3.9).

Normal plant development was only affected by cross combination (Table 3.10). A high number of plants (92.24%) were recovered from 'Lakeland' limequat while the lowest percentage was regenerated from 'Limonero Fino 49' (32.28%). Plant recovery was not affected by the pollen progenitor in 'Todo el Año' progenies (Table 3.8).

Induction of seedling development

In 2002, progenies from 'Todo el Año' x HF and 'Lisbon' x HF were numerous. In general, germination rates of small embryos were 75.11 and 68.02 % for 'Todo el Año' and 'Lisbon' embryos, respectively. Embryos with a compact-mass appearance germinated at a low rate, developing occasionally into callus. This response was more notable in 'Lisbon'. Transferring germinated embryos onto different media with low sugar did not affect the amount of normal seedlings. Neither cross combinations nor nutrient media influenced the percentage of normal seedlings. Both, 'Lisbon' and 'Todo el Año' showed high percentages of normally developed seedlings (73.75% and 72.14%, respectively). RMA medium (77.14%) showed a tendency to generate more normal plants than B5 (74.64%) and MS (67.05%).

Plant height was affected by cross combination ($P < 0.0001$) (Table 3.11); 'Lisbon' developed shorter seedlings (11.53mm) than 'Todo el Año' (18.78mm). Root length was also significantly influenced by cross combination ($P = 0.009$) and nutrient media ($P = 0.0002$) (Table 3.12). 'Lisbon' seedlings developed shorter roots (37.21mm) than 'Todo el Año' (48.70mm). The RMA medium enhanced significantly root growth (43.85mm) compared with MS (53.04mm) and B5 (31.98mm), respectively. The effect of B5 and MS on root length was similar. 'Lisbon'-derived plants showed less vigor than those from 'Todo el Año'.

Discussion

Fertility was higher in diploid-x-tetraploid crosses, although cross compatibility and seediness influenced the amount of embryos developed per cross combination. Harvesting timing of 60 days after pollination was appropriate to obtain a high number of immature embryos from 'Lakeland' limequat and 'Key' lime, 100-110 days for lemons, and approximately 90 days for the other seed progenitors. Here, the discussion is mainly focused on lemon and 'Lakeland' limequat embryo culture, owing to the larger amounts of immature embryos recovered.

Small immature embryos of 'Lakeland' limequat, 'Todo el Año' and 'Limonero Fino 49' germinated at high rates. Conversely, 'Limoneria 8A' experienced the lowest germination and plant recovery rate regardless of the developmental stages of the aborted embryos. Low competence of 'Limoneria 8A' embryos to grow *in vitro* may be associated with the medium composition and to cross incompatibility, since small progenies were only obtained from two out of four crosses. Small immature embryos germinated at a higher rate on B5, contrasting with the lack of effect of germination media on immature cotyledonary embryos. Diploid hybrids of *C. aurantium* x *Poncirus trifoliata* in heart-shaped stage were successfully rescued using a modified White's medium containing low mineral strength, casein hydrolysate and high sucrose concentration (Rangan et al. 1969).

Media with high salt strength, MS and MT are commonly used for citrus embryo culture. MT supplemented with GA, high sucrose level and malt extract was successfully applied for globular diploid embryos of *C. aurantium*. Heart stage embryos required less GA and early cotyledon responded better to MS medium with maltose (Carimi et al. 1998). Triploid lemon hybrids have been rescued on MS (Starrantino and Reforgiato-

Recupero 1981) as well as MT (Tusa et al. 1996), but the development stages of aborted embryos and germination rates were not specified in these studies.

Low-salt media improved germination and plant development in *Prunus* (Emershad and Ramming 1994) and *Carica* (Magdalita et al. 1996). Here, germination of small embryos was not significantly affected by sucrose concentration, nonetheless it was noticed that a higher germination rate occurred at 219.11 mM sucrose. High sucrose concentration generates high osmotic conditions in the medium, which avoids precocious germination of pro-embryos, and supports normal plant growth (Hirsch et al. 2001).

Small embryos showed lower competence to develop into normal plants compared to immature cotyledonary embryos under the studied culture conditions. Large numbers of globular and heart-shaped embryos germinated, showing root enlargement and slow shoot growth. Apical shoots were green, but no further growth was observed for weeks. Some embryos were induced to re-grow on DBA3 medium (Appendix H), which is rich in organic and mineral compounds and plant growth regulators.

Low plant recovery from small embryos might be related to the polyploid character of certain zygotic hybrids. Indeed, the most frequent viable aneuploids in a citrus seedling population from interploid crosses were $3X + 1 = 28$ (24.80%) and $4X + 1 = 37$ (45.90%) (Esen and Soost, 1972a). Nonetheless, a wide range of aneuploids (22-41) was more common in developing embryos. They hypothesized that the number of extra or absent chromosomes in aneuploids, as well as the type of information stored in the extra or missing chromosomes determine the plant viability and vigor (Esen and Soost, 1972a). Seedlings also experienced dieback, this physiological disorder also affected the plant survival.

Overall, nutrient media and culture conditions were appropriate for germination of immature cotyledonary embryos. The germination percentage was high for cotyledonary embryos in all cross combinations except for 'Limonero Fino 49' x HF. Very low numbers of normal plants were also recovered, suggesting that the nutrient media did not meet their requirements. The amount of normal seedlings recovered from immature embryos in the 2001 season was only significantly affected by the seed progenitor genotype, since progenies from different pollen progenitors and the same seed parent had similar percentages of plants recovered from small and immature cotyledonary embryos.

The RMA, B5 and MS media did not significantly affect seedling growth and development, nor plant recovery of germinated globular, heart-shaped and early cotyledonary stage embryos. Root length was the only variable significantly affected by media; longer roots were induced by RMA. The presence of activated charcoal and NAA in RMA medium may be responsible for longer roots. Incubation in activated charcoal promoted rooting of *Annona squamosa* shoots (Lemos and Blake 1996). Total plant recovery for 'Todo el Año' was 72.15% and 73.75 % for 'Lisbon'. A higher percentage of normal seedlings were recovered from 'Todo el Año' in 2002 compared with 2001 tests (Table 3.5).

In the 2002 experiments, the use of B5 with higher sucrose concentration (219.11) and embryo culture for 15-17 days on the germination medium might have been responsible for the difference in the amount of plant recovery between the 2001 and 2002 experiments. Embryos were incubated for longer times (4-6 weeks) on the germination media in 2001. Germination medium with high sucrose concentration and the subsequent

transfer to a medium lacking sucrose has been recommended for heart-shaped embryos of citrus (Rangan et al. 1969).

Interestingly, diploid seedlings were more vigorous than triploid seedlings in most cases, furthermore the percentages of normal diploid seedlings was higher (86.90%) than normal triploid seedlings (58.34%) in 'Todo el Año'. 'Lisbon' had similar results for normal diploid (76.44%) and triploid (72.22%) seedlings. Comparing means of normal diploid (81.55%) and triploid seedlings (65.45%) of both progenies by z-test, a significant difference ($P_{(Z<Z_c)}=0.02156$) was determined. Seedlings that did not develop into normal plant showed either abnormal development, giving rise to callus or non-growing apical meristem.

The high vigor of nucellar citrus embryos compared to zygotic embryos has been reported (Cameron and Soost 1968); this phenomenon is associated with the heterozygosity of the seed parents. A high percentage of heterozygosity has been confirmed for lemons, limes, and other citrus (Herrero et al. 1996). Citrus heterosis originates by hybridization or mutation that is naturally preserved by nucellar embryos. On the contrary, sexual embryos particularly those developed from selfing are likely to express unfavorable recessive genes that are detrimental to vigor and fertility (Cameron and Soost 1968).

Small embryos of 'Todo el Año' and 'Limonero Fino49' lemons and 'Lakeland' limequat were induced to germinate *in vitro* at a high rate. The developmental stage of embryos affected their response to the germination medium. Embryos in more advanced stages were recovered in larger amounts; however the proportion of triploids was lower owing to the rapid development of nucellar embryos. Seediness influences the amount of

triploids recovered. Plant recovery from small embryos reached a high level in the 2002 experiments. The incidence of embryos with non-growing apical meristem will require more research to determine the causes and possible solutions.

Table 3.1. Developmental stages of lemon embryos obtained from diploid x tetraploid crosses 105-110 days after pollination corresponding to the 2001 season.

Progenitors		Embryo Developmental Stage		
Female	Pollen	Globular-early cotyledonary	Immature Cotyledonary	Fully mature
Lisbon	VF	24	19	51
	KLV	38	29	9
Todo el Año	HF	108	55	89
	VF	35	20	19
	KLV	47	56	121
Limoneria 8A	HF	36	16	16
	VF	0	0	0
	KLV	0	0	0
	VRL	19	11	17
Limonero	HF	138	36	26
	VF	0	0	0
Fino 49	KLV	26	13	7
	VRL	50	20	12
Femminello	HF	31	16	10
	VF	0	0	0
Santa	KLV	0	0	0
	VRL	0	0	0
Teresa	HF	0	0	0
	VF	0	0	0
	KLV	0	0	0
	VRL	0	0	0
Arancino	HF	0	0	0
	VF	0	0	0
	KLV	0	0	0
	VRL	0	0	0
Genova	HF	0	0	0
	VF	0	0	0
	KLV	0	0	0
	VRL	10	6	3

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + Rough lemon.

Table 3.2. Developmental stages of acid-citrus fruit embryos obtained from diploid x tetraploid crosses in the 2001 season.

Progenitors		Embryo Developmental Stage		
Female	Pollen	Globular-early cotyledonary	Immature Cotyledonary	Fully mature
Key lime	HFo	22	10	6
	VF	33	15	2
	KLV	24	11	2
Lakeland limequat	HF	32	46	2
	VF	18	6	9
	KLV	16	9	8
Palestine sweet lime	HF	3	0	0
	VF	0	0	0
	KLV	12	5	0
Etrog citron	HF	17	6	193
	VF	16	62	0

KLV: 'Key' lime + 'Valencia' sweet orange; HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon. Fruit from 'Key' lime and 'Lakeland' limequat were collected 60 days after pollination and 90 days from 'Palestine' sweet lime and 'Etrog' citron.

Table 3.3. Developmental stages of embryos obtained from tetraploid x diploid crosses 90 days after pollination in the 2001 season.

Progenitors		Embryo Developmental Stage		
Female	Pollen	Globular-early cotyledonary	Immature Cotyledonary	Fully mature
HF	Key lime	5	7	0
	Lakeland limequat	3	2	0
VF	Key lime	0	0	0
	Lakeland limequat	0	0	0
VRL	Key lime	0	0	0
	Lakeland limequat	0	0	0

HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + 'Rough lemon.

Table 3.4. Analysis of variance of percentage germination of small embryos rescued from diploid-x-tetraploid crosses completed in th2 2001 season.

Source of Variance	DF	SS	MS	F Value	Pr>F
Cross	6	9094.68	1515.78	2.80	0.0155
Medium	1	3033.79	3033.79	5.61	0.0201
Sucrose	1	1092.78	1092.78	2.02	0.1588
Cross x Medium	6	4279.01	713.17	1.32	0.2577
Cross x Sucrose	6	582.83	97.14	0.18	0.9817
Medium x Sucrose	1	89.22	89.22	0.16	0.6856
Error	85	45970.44			

Table 3.5. Mean percentages of small embryo germination and normal seedling recovery from small embryos.

Cross	Germination (%)	Normal Plant (%)
Todo el Año x KLV	85.00 a	39.58 ab
Todo el Año x VF	84.36 a	34.24 b
Todo el Año x HF	82.91 a	37.32 b
Limonero Fino 49 x VRL	79.17 a	41.39 ab
Limonero Fino 49 x HF	78.65 a	39.50 b
Lakeland limequat x HF	75.00 a	64.73 a
Limoneria 8A x HF	52.78 b	11.11 c

Mean followed by different letters within each column indicates significant difference ($P < 0.05$) by Fisher's least significant difference (LSD) procedure. HF: 'Hamlin' sweet orange + 'Femminello' lemon; VF: 'Valencia' sweet orange + 'Femminello' lemon; VRL: 'Valencia' sweet orange + Rough lemon. % Normal Plant: germinated embryos that developed into seedling with growing stem and root.

Table 3.6. Analysis of variance of normal seedling percentage recovered from small embryos obtained from diploid-x-tetraploid crosses.

Source of Variance	DF	SS	MS	F Value	Pr>F
Cross	6	14734.91	2469.59	5.54	0.0269
Medium	1	871.99	12.60	0.01	0.3465
Sucrose	1	510.82	41.68	0.04	0.4707
Cross x Medium	6	6920.71	1141.94	1.17	0.3218
Cross x Sucrose	6	11479.89	1943.19	2.00	0.0794
Medium x Sucrose	1	850.91	850.91	0.87	0.3523
Error	106	82702.08			

Table 3.7. Analysis of variance of percentage germination of immature cotyledonary embryos obtained from diploid-x-tetraploid crosses.

Source of Variance	DF	SS	MS	F Value	Pr>F
Cross	3	9119.08	3039.69	36.34	<0.0001
Medium	1	8.25	8.25	0.10	0.7551
Sucrose	1	1289.88	1289.88	15.42	0.0003
Cross x Medium	3	98.03	32.68	0.39	0.7604
Cross x Sucrose	3	3173.24	1057.75	12.64	<0.0001
Medium x Sucrose	1	114.09	114.09	1.36	0.2498
Error	40	3346.27			

Table 3.8. Mean of germination and normal seedling percentages of immature cotyledonary embryos from diploid-x-tetraploid crosses.

Cross	Germination (%)	Normal plant (%)
Todo el Año x KLV	100.00 a	60.02 b
Todo el Año x HF	98.44 a	65.00 b
Lakeland limequat x HF	97.98 a	92.24 a
Limonero Fino 49 x HF	65.30 b	32.28 c

Mean followed by different letters within each column indicates significant difference ($P < 0.05$) by Fisher's least significant difference (LSD) procedure. KLV; 'Key' lime + 'Valencia orange'; HF: 'Hamlin' orange + 'Femminello' lemon. %Normal plant; germinated embryos that developed into seedling with growing stem and root.

Table 3.9. Mean of germination percentage of immature cotyledonary embryos determined by the sucrose-cross interaction.

Cross	Sucrose mM	Germination (%)
Lakeland limequat x HF	146.07	95.83 a
Lakeland limequat x HF	204.50	100 a
Todo el Año x KLV	146.07	100 a
Todo el Año x KLV	204.50	100 a
Todo el Año x HF	146.07	100 a
Todo el Año x HF	204.50	96.87 a
Limonero Fino 49 x HF	146.07	45.50 b
Limonero Fino 49 x HF	204.50	85.09 a

Mean followed by different letters within each column indicates significant difference ($P < 0.05$) by Fisher's least significant difference (LSD) procedure. KLV; 'Key' lime + 'Valencia orange'; HF: 'Hamlin' orange + 'Femminello' lemon.

Table 3.10. Analysis of variance of normal seedling percentage recovered from immature cotyledonary embryos-obtained from diploid-x-tetraploid crosses.

Source of Variance	DF	SS	MS	F Value	Pr>F
Cross	3	18702.51	6234.17	7.86	0.0003
Medium	1	85.72	85.71	0.11	0.7441
Sucrose	1	849.24	849.24	1.07	0.3071
Cross x Medium	3	1284.06	428.02	0.54	0.6579
Cross x Sucrose	3	1216.92	405.64	0.51	0.6767
Medium x Sucrose	1	33.81	33.81	0.04	0.8375
Error	39	30923.29			

Table 3.11. Analysis of variance of plant height of plants recovered from rescued-immature embryos generated in diploid-x-tetraploid crosses.

Source of Variation	DF	SS	MS	F Value	Pr>F
Cross	1	2228.89	2228.89	18.15	<.0001
Medium	2	193.77	96.88	0.68	0.5078
Cross x Medium	2	70.29	35.15	0.25	.7817
Error	203				

Table 3.12. Analysis of variance of root length of plants recovered from rescued-immature embryos generated in diploid-x-tetraploid crosses.

Source of Variance	DF	SS	MS	F Value	Pr>F
Cross	1	6485.34	6485.34	7.85	0.0056
Medium	2	14888.28	7444.14	9.02	0.0002
Cross x Medium	2	128.39	64.20	0.08	0.9252
Error	193				

CHAPTER 4 GERMINATION INDUCTION OF ABORTED TRIPLOID HYBRID MANDARIN EMBRYOS

Introduction

Genetic improvement of mandarin, like many other citrus, is aimed to develop new seedless cultivars as the primary goal. To accomplish that goal interploid hybridization, diploid x tetraploid or the reciprocal, is routinely applied among other approaches to produce triploid hybrids (Grosser et al. 2000). Triploids are sterile, therefore their fruit have no seeds or very few. In tetraploid x diploid crosses, the proportion of triploids was higher from sexual tetraploid female progenitors than the observed with partial polyembryonic tetraploid female parents, or when the tetraploid was the pollen progenitor (Cameron and Burnett 1978). However, the development of citrus seeds from interploid crosses in either direction is affected by the ploidy ratio between embryo and endosperm (Esen and Soost 1973). On the contrary, the endosperm failure in interploid, intraspecific and interspecific crosses has been explained by the endosperm balance number hypothesis that considers the maternal-paternal genome ratio of the hybrid endosperm (Johnston et al. 1980).

Embryo culture is a useful technique in mandarin breeding programs that include interploid crosses as an approach, since new hybrids might not be able to survive in most cases owing to post-zygotic incompatibilities. Successful *in vitro* culture of aborted embryos relies on a suitable nutrient medium for embryo germination and further seedling growth, embryo age, genetic background of the new hybrid, and an efficient

excision technique. Although nutrient requirements vary from species to species, it is established that a developing embryo has different requirements during maturation (Ramming 1994). In citrus, the most commonly used media for embryo culture have been Murashige and Skoog (MS) (1962) and Murashige and Tucker (MT) (1969). Both media contain the same composition and concentrations of mineral salts but differ on the higher concentrations of nicotinic acid, pyridoxine x HCl, and thiamine x HCl present in MT. Diploid citrus hybrids were rescued at very early stages when low salt strength medium (modified White's medium) supplemented with casein hydrolysate was utilized (Rangan et al. 1969).

Malt extract and GA₃ are commonly used addenda in citrus embryo cultures; however, low germination rates have been reported for different monoembryonic and polyembryonic female progenitors (Deng et al. 1996; Khan et al. 1996). Conversely, GA induced a very high rate of germination (95.50%) of aborted triploid mandarin embryos (Oiyama et al. 1991; Sykes and Lewis 1996).

The principal objective of this research was to study the effect of sucrose, GA and nutrient media as well as their interaction on germination of aborted embryos of mandarin collected from interploid crosses 90 days after pollination.

Materials and Methods

Plant Material

A diploid-monoembryonic mandarin hybrid, LB8-9 ((Clementine (*Citrus clementina* Hort. Ex Tanaka)) x Minneola tangelo (Duncan grapefruit x Dancy tangerine)), was hand-pollinated with pollen from the allotetraploid somatic hybrid Nova mandarin hybrid + Succari orange. Plant progenitors were grown at the CREC-IFAS-University of Florida, USA.

Pollination

The methods utilized for pollination and surface sterilization of fruit are described in chapter 2.

Embryo Excision

Ninety-day-old fruit were surface-sterilized following the procedure described in chapter 2. Under aseptic conditions, seeds were extracted and grouped into small and medium sizes. The number of empty seeds was also recorded. Embryos were carefully excised under 100X magnification using a stereomicroscope.

Germination Induction Treatments

Eighteen media resulting from combinations of two nutrient media: EME (Appendix A) and Gamborg's B5 basal medium (Gamborg et al. 1968); and three concentrations of sucrose: 146.07, 204.50 and 262.93 mM and gibberellic acid (GA_3): 0, 4.54 and 9.09 μ M were evaluated. All media contained 0.5 g/L malt extract. Filtered-sterilized GA_3 was added to warm ($\sim 55^\circ\text{C}$) autoclaved media, immediately afterwards, 50 mL was poured into Petri plates (100x20 mm). The pH of all media was adjusted to 5.8 using 1N KOH, prior to the addition of 7 g/L agar (Sigma Chemical Co., St. Louis, MO). Five embryos were plated per Petri plate, and then the plate was sealed with Nescofilm (Karlson Research Products Corporation, Santa Rosa, CA). Embryos were grown at 28°C , under cool white fluorescent tubes with continuous light exposure.

Data Analysis

A 2×2^3 factorial experiment in a completely randomized design was applied using five embryos per Petri plate, and 6 plates per treatment. Embryo germination was evaluated a month after the experiment initiation. Fisher's least significant difference (LSD) procedure was applied to determine differences among means. Statistical analysis was performed with SAS (SAS Institute, Cary, NC).

Results

The average of fruit diameter at 90 days after pollination was 4.32 cm. Although, fruits were seedy (20.24seeds/fruit), high numbers of seeds were empty; 47.67% for medium size and 92.86% for small ones. Endosperm was entirely degenerated in most of the seeds, even though liquid endosperm was eventually observed. Most of the embryos were very compact and small. A few translucent globular embryos were rescued, but none of them germinated. Germination of aborted embryos was significantly affected ($P<0.0001$) by culture medium and GA (Table 4.1). Germination was higher on B5 (62.22%) than on EME (45.02%). The supplementation of culture medium with GA at either 4.54 or 9.09 μM increased germination significantly (65.56% and 66.98% respectively), these responses differed significantly ($P<0.0001$) from the treatment containing no GA (28.33%).

Regarding the interaction effects, significant differences ($P=0.0007$) were obtained for sucrose-GA interactions. At the lowest sucrose concentration, germination improved with increases of GA levels, and similar trend was observed at 204.50 mM sucrose, with higher rates (Figure 4.1). A different trend was observed at 262.93 mM sucrose, as the germination rate only increased at 4.54 μM GA. The highest GA concentration reduced the rate of germination when sucrose level was 262.93 mM. In the absence of GA, a high number of aborted embryos developed into either solid callus or compact embryonic callus. Some small seedlings developed secondary embryos.

Discussion

Most of the excised embryos were amorphous and difficult to separate from the inner seed coat, making it difficult to identify their developmental stage. Histological analysis of monoembryonic citrus seeds resulting from 2X x 4X crosses showed that

embryos did not differentiate normally into plumula-radicle axis and cotyledons, as a consequence of early degeneration of endosperm. Aborted embryos usually developed into structures of different sizes and shapes (Esen and Soost 1973). The higher percentage of small empty seeds might be associated with very early embryo abortion due to lethal polyploid configuration. It was determined that empty and partially empty citrus seeds arise from fertilized ovules (Esen and Soost 1971).

In this study, aborted LB8-9 embryos germinated more efficiently on B5, a medium that has been reported for embryo culture in other crops as well (Sukno et al. 1999; Sharma and Kumar 1996; Qi et al. 1998; Bodanese-Zanettini et al. 1996). Comparing the basic composition of B5 and EME, the former has lower concentrations of salts, nicotinic acid and pyridoxine. Different media with lowered salt strength have been successfully applied for rescuing citrus pro-embryos (Rangan et al. 1969), as well as peach (Emershad and Ramming 1994) and papaya embryos (Magdalita et al. 1996).

Considering the positive GA effect, germination of aborted LB8-9 embryos improved with the addition of GA to the medium, although 4.54 and 9.09 μM induced similar responses. *In vitro* germination of citrus diploid pro-embryos was enhanced by GA, germination of globular and heart-stage embryos was best at 14.4 and 2.9 μM GA respectively, while early cotyledon staged embryos germinated better on medium supplemented solely with malt extract and sucrose (Carimi et al. 1998). However, it was noticed that the rate of germination of early cotyledon embryos on malt extract was similar to the response induced by 2.9 μM GA. Triploid hybrids of *Citrus clementina* x *Poncirus trifolita* germinated at high rate when 1 mg/L GA was used for embryos collected at maturity (Oiyama et al. 1991).

The dual effect of endogenous GA on germination has been identified as reserve food mobilization and embryo growth (Karssen et al., 1989). During seed development, the active GAs are more abundant in immature embryos. In absence of GA, embryos mostly developed into embryogenic calli with very small green embryos rising compactly, similar structures were eventually observed on treatments with GA. Non-embryogenic calli also developed. Secondary embryos were observed on small seedling growing on media with any GA level. Embryogenic callus formation was also reported for Kinnow mandarin (Khan et al. 1996). Development of somatic embryos corroborates the high embryogenic potential of immature embryos (Nerderwieser 1990).

Germination of LB8-9-aborted embryos was also affected by the sucrose-GA interaction (Fig. 4.1). The lowest sucrose level induced poor germination, perhaps because the energy source was limited. On the contrary, dramatic increases in germination rate occur at higher sucrose levels with the addition of GA. It has been proven that sucrose has two major roles in embryo culture; namely nutritional and osmotic. Very young embryos require higher osmolality than more advanced ones (Rietsema et al. 1953; Niederwieser et al 1990). High osmotic pressure reduces growth of pro-embryos, avoiding precocious germination and allowing reserve accumulation.

In diploid citrus, a good number of early cotyledonary embryos germinated well at 60 mM sucrose, however 150 mM was more effective, inducing higher plant recovery. Furthermore, the addition of GA to the nutrient media improved significantly the sucrose effect on citrus germination (Carimi et al. 1998), which agrees with the results of this study. Similarly, Sykes and Lewis (1996) also reported efficient germination for diploid hybrids using GA with a high sucrose concentration. In general, a high sucrose level is

used as an initial step for pro-embryos; subsequently it is lowered to induce plant growth (Rangan et al. 1969; Ramming 1990; Chandler and Beard, 1983). Numerous factors influence *in vitro* germination of aborted embryos. In this particular case, an earlier harvest after pollination may facilitate embryo excision. All three studied factors affect germination; however, sucrose effect was influenced by GA concentration.

Table 4.1. Analysis of variance of germination percentage of rescued embryos from interploid crosses of LB8-9 mandarin x 'Nova' mandarin + 'Succari' orange

Source of Variation	df	SS	MS	F value	Pr>F
Medium	1	7902.30	7902.30	17.31	<.0001
Sucrose	2	239.96	119.98	0.26	0.7695
GA	2	34372.25	17186.13	37.64	<.0001
Medium x Sucrose	2	1503.85	751.92	1.65	0.1982
Medium x GA	2	705.59	352.79	0.77	0.4647
Sucrose x GA	4	9700.85	2425.21	5.31	0.0007
Error	93	42459.69			

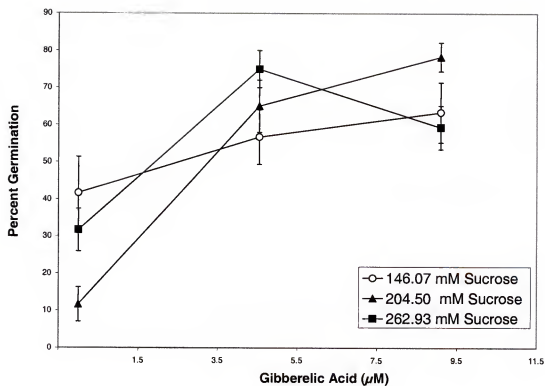


Figure 4.1. Mean of percentage germination of aborted LB8-9 mandarin embryos influenced by the GA- sucrose interaction

CHAPTER 5
A STOMATAL INOCULATION METHOD FOR SCREENING TRIPLOID HYBRIDS
OF LAKELAND LIMEQUAT FOR RESISTANCE TO ASIATIC CITRUS CANKER
(*Xanthomonas axonopodis* pv. *citri*)

Introduction

Xanthomonas axonopodis pv. *citri* strain A causes the most serious leaf and fruit spotting bacterial disease of citrus, known as Asiatic citrus canker (ACC). Strain A is virulent to all citrus species (Koizumi, 1981) and induces infection responses that range from highly susceptible to highly resistant. 'Mexican' lime (*Citrus aurantifolia* (Christm.) Swing.), grapefruit (*Citrus paradisi* Mac.) and certain sweet oranges (*Citrus sinensis* (L.) Osb. are among the most susceptible commercial varieties to ACC. Chemical control and cultural practices are part of integrated management systems applied in countries where ACC is endemic; nevertheless Gottwald et al. (2002) stated that it is probably less profitable to grow susceptible citrus varieties in tropical and sub-tropical climates in the presence of ACC, as a consequence of the high production costs linked with windbreaks and additional foliar sprays of copper bactericides.

Citrus breeding is the best long-term solution for management of ACC. New varieties with greater ACC resistance can be obtained by introgressing canker resistance genes from more resistant citrus species or its relatives into susceptible ones. Resistance to ACC can be found in calamondin (*Citrus madurensis* Lour.), kumquat (*Fortunella margarita* (Lour.) Swing.) (Schubert et al. 2001; Reddy 1997) and wide citrus relatives belonging to the genera *Atalantia*, *Severinia*, *Feronia*, and *Microcitrus* (Reddy 1997) that have been reported as ACC field resistant. Conventional citrus breeding for disease

resistance in scion germplasm is considered difficult since the resistance is found in citrus relatives, whose genetic makeup might interfere with the expressions of traits related to fruit quality and production, in addition to complications imposed by long life cycles (Soost and Roose 1996). Moreover, progenies from intergeneric crosses are generally small and/or perform poorly as a consequence of lethal recombinations (Barrett, 1985). Nonetheless, 'Lakeland' limequat, an intergeneric hybrid between ACC susceptible, 'Mexican' ('Key') lime (*Citrus aurantifolia* (Christm.) Swing.) and highly resistant kumquat (*Fortunella japonica* (Thumb.) Swing.) progenitors, was identified as highly resistant after field evaluations of naturally infected budded trees and seedlings (Reddy, 1997). Some interspecific Nepali hybrids (*Citrus aurantifolia* x *Citrus medica*) were ACC free after three-year evaluation in an infested field (Prasad, 1997).

'Lakeland' limequat is a hybrid that bears Mexican-acid-lime type fruit and also shows more cold hardiness than its female progenitor. The new acid-citrus breeding program at the CREC-University of Florida-USA has generated a total of 25 new Lakeland limequat triploid hybrids through interploid crosses with allotetraploid somatic hybrids and autotetraploids during the 2000-2001 seasons (Viloria et al. unpublished). Currently a large number of citrus allotetraploid somatic hybrids are available for use in scion breeding programs (Grosser et al. 1998; 2000). These allotetraploid parents can be used to increase the genetic diversity of new progenies.

Successful and timely screening of new citrus progenies for ACC resistance requires an accurate and repeatable inoculation technique that yields results in a short time. Screening for ACC resistance under natural infection conditions has been carried out in countries where this disease is widespread (Prasad et al. 1997; Reddy 1997;

Zubrzycki and Diamante de Zubrzycki 1981), but this approach cannot be applied in countries where ACC is not endemic. Several artificial inoculations techniques have been applied for bacterial inoculation of citrus as follows: wound inoculation of mesophyll tissue using pinprick technique (Graham et al. 1990; Gottwald et al. 1993; Prasad et al. 1997); leaf abrasion (Din Kahn and Hingorani 1970); injection infiltration (Stall et al. 1980; 1982); and inoculation of wounded and non-wounded mid vein of *in vitro* plants (Lopez and Navarro 1981).

Zubrzycki and Diamante de Zubrzycki (1981) determined that natural infection and induced infection by injection-infiltration inoculation were not always related. Leaf characteristics associated with cuticle development and wax deposition influence inoculum infiltration in the leaf, while the bacterial growth in the intercellular spaces of mesophyll is determined by mesophyll cell resistance. External and mesophyll resistances change with leaf age (Graham et al. 1992). Pinprick inoculation, which bypasses external resistance, induces general susceptibility in citrus and citrus relatives that are known to perform differently in field conditions due to circumvention of stomatal infection by wounding (Gottwald et al. 1993). Nevertheless, mesophyll resistance is an important factor in screening for resistance to ACC (Stall et al. 1982). A stomatal inoculation apparatus designed to precisely infiltrate the bacterial inoculum with no leaf injury (Gottwald and Graham 1992), allowed for detailed assessment of the infection response without a wounding effect. In their study, there were significant differences in bacterial growth and number of lesions that developed from stomatal infection among citrus varieties known to vary in field resistance to ACC (Graham et al. 1992).

The present research was undertaken to validate an ACC resistance screening protocol by applying a modification of Gottwald-Graham methodology for stomatal infiltration with low density of bacteria on triploid hybrids of 'Lakeland' limequat and their progenitors under greenhouse conditions. The accuracy of the injection infiltration inoculation technique over repeated trials was determined by Spearman's rank correlation analysis of lesion number and population density in the leaf mesophyll.

Materials and Methods

Four separate ACC screening assays were performed on the same plant material at a quarantine facility of the Florida Department of Agriculture and Consumer Services- Division of Plant Industry (DPI), Gainesville, Florida in May, July, August and November 2002.

Plant Material

Nine one-year-old triploid hybrids of Lakeland limequat (*Citrus aurantifolia* x *Fortunella japonica*) were selected for this study along with the Lakeland limequat seed parent and the following pollen parents: the autotetraploids 'Femminello' lemon (*Citrus limon* (L.) Burm. f.) and 'Giant Key' lime (*C. aurantifolia*), and two allotetraploids somatic hybrids 'Key' lime + 'Valencia' orange and 'Hamlin' orange + 'Femminello' lemon. Two morphologically different Lakeland limequat autotetraploids were also tested. These autotetraploid 'Lakeland' limequats were obtained from interpid crosses completed in the 2000 season (chapter 2). To identify the origin of these tetraploids RAPD-PCR analysis was carried out. Seven out 24 primers (decamer and twelve-mer primers) showed polymorphism between progenitors, but the DNA patterns of the autotetraploid were identical to the seed progenitor ('Lakeland' limequat).

'Meiwa' kumquat (*Fortunella crassifolia* Swing.) and 'Pineapple' sweet orange were included in the evaluation as resistant and susceptible standards, respectively. Budded plants were grown in 4x4x14" plastic pots (IEM Plastics (PTY), Ltd.) containing Metro Mix 500 (The Scotts Co., Marysville, OH). Mineral nutrition was provided periodically with Essential Minor Elements (Agricultural Insecticides, Inc. Palmetto, FL) and Osmocote (The Scotts Co., Marysville, OH). Lateral shoots were removed in order to keep a single strong stem. Plants were cut back, keeping 1-2 nodes above the bud union. Scions were trained to one shoot to maintain uniformity of plant growth. Three to four weeks after shoot removal, there were sufficient newly expanding leaves for inoculation. After each experiment, trees were cut back to induce new growth flushes as needed for repetition of the experiment.

Inoculum and Inoculation Technique

The primary source of inocula was cultures of *Xanthomonas axonopodis* pv. *citri* X02-007 from Broward Co. growing on nutrient agar. For inoculation, cells in late log phase were isolated from bacteria grown in nutrient broth medium that were incubated for 24 hours on a rotatory shaker at 28-29 °C. After centrifugation of the suspension, the bacterial pellet was resuspended in 0.075 M phosphate buffer (pH 7.0) and cell concentration was adjusted to 10^3 and 10^4 cfu/mL using a spectrophotometer at 620 nm. Bacterial concentration was confirmed by dilution plate counting on nutrient agar.

Young, expanding leaves with size approximately at 75 to 100% of full development were inoculated using a sterile syringe (1 cc) with no needle. Inoculum was slowly infiltrated by pressing the needle-less syringe against the abaxial leaf surface and placing a finger on the opposite side for counter-pressure, to induce a zone of water-soaking tissue 2 mm beyond the diameter of the syringe opening. The inoculation site

was approximately 6 mm diameter. Three injection infiltrations were performed on the leaf blade on each side of the mid vein. After wiping off the excess of inoculum, the inoculated shoots were covered with plastic bags for 2-3 days, in order to maintain high humidity favorable for bacterial growth in the leaves. Experiments were completed in a greenhouse with temperature adjusted to 27/23°C (day/night) and natural photoperiod. Inoculated plants were rotated on the bench greenhouse twice a week.

Disease Assessment

The number of lesions per inoculation site and bacterial population per lesion were recorded approximately two weeks after inoculation. Lesion number was counted on inoculated leaves using a 10X magnifying hand glass. Inoculated sites for sampling of bacterial population were chosen according to the mean number of lesions per site per hybrid. Inoculation sites were excised from the leaf with a cork borer and ground individually in 2 mL phosphate buffer (pH 7.0). Serial dilutions: 10^2 , 10^3 , 10^4 and 10^5 were spread on replicate plates containing KCB, which is semi-selective medium for xanthomonads. The KCB medium consisted of 23 g/L Difco nutrient agar, 5 g/L glucose, and the antibiotics Kusugamycin, Cephalexin, and Bravo at 16 mg/L each. Three to four days after incubation, colonies were enumerated as the mean cfu/mL from replicate samples.

Data Analysis

All experiments were performed in a completely randomized experimental design. The analysis of variance was applied according to a Mixed Model procedure for a nested experiment (SAS Proc Mixed Model, Cary, NC). Lesion number/inoculation site was recorded in each experiment and bacterial population/lesion was evaluated in the last two assays. The bacterial counts were $\log_{10}$ transformed to stabilize variances. The

difference among genotypes for infection reaction was determined by Waller-Duncan- k-ratio procedure at the 0.05% confidence level. Correlations based on the genotype ranks for lesion number and bacterial population per lesion were determined by Spearman's rank order correlation coefficient r_s (Ott, 1993).

Results

Consistent differential infection responses of the tested genotypes to *X. axonopodis* pv. *citri* could not be discerned at the inoculum concentration of 10^3 cfu/mL. The coefficients of variance of the means for genotypes were high, ranging between 57.08 and 75.12. Therefore, the 10^4 cfu/mL inoculum concentration was used in subsequent evaluations. Leaf mesophyll inoculation using stomatal infiltration technique induced well-developed ACC lesions fifteen days after inoculation. Lesions were localized at the inoculation site in most cases. Differences in lesion appearance between resistant and susceptible plants were noted. Resistant 'Meiwa' and 'Lakeland' limequat cultivars developed lesions that were small, orange to brown in color, and did not erupt through the leaf cuticle (Figures 5.2 A and B). Similar symptoms were observed on the two autotetraploid 'Lakeland' limequats (Figures 5.2 E and F) and the triploid hybrids 'Lakeland' limequat x ('Key' lime + 'Valencia' orange) and 'Lakeland' limequat x ('Hamlin' sweet orange + 'Femminello' lemon) and 'Lakeland' limequat x 'Giant Key' lime. Susceptible genotypes developed lesions that erupted through the leaf cuticle and continued to expand and eventually coalesce (Figures 5.1 F and G).

Lesion numbers/inoculation site in individual assays were significantly different among genotypes in the ACC infection reaction induced in May, July and August. Consistently the known susceptible genotype 'Pineapple' sweet orange was significantly different from the resistant 'Meiwa' kumquat (Table 5.1). The hybrid from 'Giant Key'

lime and those from 'Hamlin' orange + 'Femminello' lemon as the seed parent developed high number of lesions and ranked closer to the pollen progenitors. Other hybrids developed fewer lesions than their pollen parents and always ranked closer to 'Lakeland' limequat (diploid and autotetraploid genotypes) and 'Meiwa' kumquat. Only in the November assay was the difference among genotypes not significant ($P=0.06$). Nonetheless; the resistant 'Lakeland' limequat and 'Meiwa' ranked lowest in lesion number.

Analysis of the four experiments simultaneously, considering lesion number and the fifteen genotypes common to all four assays, showed significantly strong correlations among the genotype rankings obtained in May and the subsequent three assays according to the Spearman's rank (Table 5.2). Less significant and weak relationships were observed when the ranking recorded in November was compared with July ($r_s=0.44$, $P=.098$) or August ($r_s=0.52$, $P=0.046$).

Highly significant differences in bacterial population per lesion (cfu/lesion) were also observed among genotypes. The highest bacterial population was recorded from 'Pineapple' sweet orange and the lowest from 'Meiwa' kumquat in the August experiment (Table 5.3). A slight variation of these results was recorded from trees inoculated in November. The correlation coefficient between genotype rankings obtained from the two bacterial population studies was highly significant ($r_s = 0.6082$, $P < 0.0001$). The relationship between lesion number and bacterial population per lesion was also significant for the tests conducted in August and November. Comparing genotype rankings of lesion number vs bacterial population, the relationship was more highly significant in August ($r_s = 0.59$, $P= 0.0094$) than in November ($r_s= 0.55$; $P= 0.0217$).

Discussion

In this study, injection infiltration of *X. axonopodis* pv. *citri* at 10^4 cfu/mL induced differential bacterial population and lesion responses among genotypes more consistently than at a concentration of 10^3 cfu/mL. Although 10^3 cfu/mL has been previously used as inoculum concentration to evaluate mesophyll resistance (Stall et al 1982), much higher concentrations have also been applied for studies of leaf mesophyll resistance mechanisms or screening using different inoculation methods than injection infiltration (Gottwald et al. 1993; Dienelt and Lawson 1989; López and Navarro 1981).

Here, stomatal inoculation using a syringe with no needle induced lesion number for 15 closely related citrus genotypes that were well correlated among four assays. Somewhat lower rank correlation coefficients between summer and fall experiments may be related to environmental factors. Although these experiments were performed in a greenhouse, uncontrolled environmental variables such as shorter photoperiod and cooler temperatures in November may have influenced the ACC lesion development. These environmental conditions may affect plant growth and consequently bacterial development. In November, lesions were not fully developed by 15 days after inoculation in many plants, which made it necessary to extend the incubation period for 5 more days. Yet, the ranking among genotypes remained consistent with the canker tests.

Even under varying experimental conditions, mesophyll resistance (Stall et al. 1982) seems to have played the more important role in the difference observed among genotypes. There was reasonably high consistency in genotype rankings despite variability in leaf development of the various genotypes. Expanding leaves were carefully chosen for inoculation to ensure they were at similar physiological and morphological stage of development.

Bacterial population growth in leaves also supported the concept of mesophyll resistance being different among susceptible and resistant genotypes. Genotype rankings according to lesion number were clearly related to bacterial population in the leaves; therefore both variables could be used to assess leaf mesophyll resistance. Similarly, Graham et al. (1992) found correlation between rankings of citrus genotypes based on lesion numbers and bacterial population seven days after stomatal inoculation of a diverse group of citrus genotypes. In the previous study, correlation coefficients were between bacterial population and lesion were higher for resistant than susceptible cultivars. Graham et al. (1992) furthermore reported that bacterial growth slowed down after 48-72 hours in resistant cultivars but continued in susceptible ones. Koizumi (1981) also reported slower lesion extension and lower populations in leaves of resistant plants compared to susceptible ones.

Bacterial population study is a precise method to determine quantitative resistance, but the logistics of performing repeated assays at a remote quarantine laboratory is not practical. Lesion number was a reliable quantitative and convenient measure of resistance to ACC without further measurement of bacterial population.

'Lakeland' limequat is a very promising seed parent for breeding acid citrus fruit that is resistant to canker. 'Lakeland' limequat autotetraploids and the triploid hybrids: Lakeland limequat x 'Key' lime + 'Valencia' orange and 'Lakeland' limequat x 'Hamlin' orange + 'Femminello' lemon also showed potential useful level of resistance. This research indicates that canker resistance genes from kumquat can be readily moved into acid-fruit hybrids by conventional breeding. Such an approach may also have potential in mandarin improvement. It is worth mentioning that two hybrids (GB1 and GB2) from

'Lakeland' limequat as a pollen parent (rather than seed parent) showed higher susceptibility. Continued evaluation of the ACC resistant triploid hybrids identified in this research for horticultural attributes could yield an acceptable canker resistant lime-type fruit tree to replace traditional lime trees in dooryards in south Florida that are being destroyed as part of the canker eradication program (Schubert et al. 2001).

Table 5.1. Mean of lesion number/inoculation site induced by stomatal inoculation of *Xanthomonas axonopodis* pv. *citri* on 'Lakeland' lime hybrids in four repeated assays.

Genotype	May	July	August	November
Giant Key Lime	---	---	17.56 a	13.77 ab
GB1	19.36 a	20.17 abcd	15.46 ab	23.36 a
B4-3	17.33 ab	22.95ab	10.08 abc	23.56 a
B3	16.61 ab	19.40 bcde	13.10 ab	22.92 a
GB2	16.37 ab	17.90 bcdef	12.00 ab	17.97 ab
Hamlin + Femminello	15.72 abc	27.28 a	12.30 ab	14.63 ab
4X Femminello	13.97 abcd	24.71 ab	17.74 a	13.53 ab
Key + Valencia	13.79 abcd	20.51 abcd	12.85 ab	16.05 ab
Pineapple orange	12.97 abcde	---	11.73 ab	16.25 ab
GB3	12.41 bcde	22.63 ab	12.38 ab	11.99 ab
B4-2	12.22 bcde	21.62 abc	11.96 ab	16.27 ab
4X Lakeland limequat	11.11 bcde	8.09 gf	11.73 ab	15.58 ab
B4-1	10.56 bcde	12.85 efg	10.48 abc	11.91 ab
B1	9.00 cde	---	9.67 abc	---
B2	8.52 de	14.45 cdefg	9.33 abc	14.25 ab
Lakeland limequat	7.78 de	11.17 fg	7.17 bc	8.722 b
*4XLakeland limequat	6.44 ef	10.96 fg	7.59 bc	8.18 b
Meiwa kumquat	0.93 f	13.36 defg	3.11 c	8.72 b

Different letters within each month indicates significant difference ($P < 0.05$) by Waller-Duncan K ratio procedure. B1 = 'Lakeland' limequat x 'Key' lime + 'Valencia' orange; B2 = 'Lakeland' limequat x 'Hamlin' orange + 'Femminello' lemon; B3 = 'Lakeland' limequat x 'Giant Key' lime; B4 = 'Lakeland' limequat x 4X Femminello, 1,2,3 indicate different hybrids; GB = 'Hamlin' orange + 'Femminello' lemon; x 'Lakeland' limequat, 1,2,3 indicate different hybrids.* Morphologically different autetraploid 'Lakeland' limequat.

Table 5.2. Spearman's rank order correlation coefficients among genotype rankings according to lesion number/inoculation site after fifteen days of stomatal inoculation of *Xanthomonas axonopodis* pv. *citri*.

	May	July	August
July	0.64643 P = 0.0092		
August	0.75000 P = 0.0013	0.60714 P = 0.0164	
November	0.86071 P < 0.0001	0.44286 P = 0.0983	0.52143 P = 0.0462

n=15 genotypes

Table 5.3. Mean of bacterial population/ lesion induced by stomatal inoculation of *Xanthomonas axonopodis* pv. *citri* on 'Lakeland' limequat hybrids in two repeated assays.

Genotype	August	November
	Log ₁₀ (cfu/lesion)	Log ₁₀ (cfu/lesion)
Pineapple orange	5.23 a	4.55 abc
Giant Key lime	5.06 ab	4.15 bcd
4X Femminello	4.69 abc	4.34 abcd
GB1	4.66 abc	4.46 abc
B4-3	4.59 abcd	4.74 a
Hamlin + Femminello	4.58 abcd	4.57 abc
B4-1	4.54 abcd	4.61 ab
GB2	4.44 abcd	4.56 abc
B2	4.43 abcd	4.50 abc
Key lime + Valencia	4.34 cbd	4.75 a
B4-2	4.05 cde	3.93 de
GB3	4.05 cde	4.07 cde
B3	3.96 cde	4.34 abcd
4X Lakeland limequat	3.75 de	3.85 de
B1	3.33 e	---
*4X Lakeland limequat	3.33 e	3.58 ef
Lakeland limequat	3.31 e	2.86 g
Meiwa kumquat	2.38 f	3.19 fg

Different letters within each month indicates significant difference ($P < 0.05$) by Waller-Duncan K ratio procedure. B1 = 'Lakeland' limequat x 'Key' lime + 'Valencia' orange; B2 = 'Lakeland' limequat x 'Hamlin' orange + 'Femminello' lemon; B3 = 'Lakeland' limequat x 'Giant Key' lime; B4 = 'Lakeland' limequat x 4X 'Femminello', 1,2,3 indicates different hybrids; GB = 'Hamlin' orange + 'Femminello' lemon x 'Lakeland' limequat, 1,2,3 indicates different hybrids.

* Morphologically different autetraploid 'Lakeland' limequat.

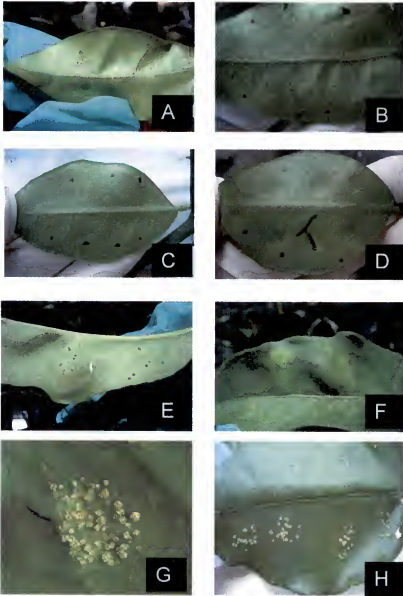


Figure 5.1. Phenotype of ACC lesions developed two weeks after inoculation. A= 'Meiwa' kumquat; B= 'Lakeland' limequat; C='Lakeland' limequat x 'Giant Key' lime; D='Lakeland' limequat x 'Key' lime + 'Valencia' sweet orange; E and F=Autotetraploid 'Lakeland' limequat abaxial and adaxial leaf surface; G and H= 'Hamlin' sweet orange + 'Femminello' lemon abaxial and adaxial leaf surface.

CHAPTER 6

SOMATIC HYBRIDIZATION BETWEEN *Citrus limon* (L.) BURM. F. AND OTHER CITRUS SPECIES: REGENERATION OF PROTOPLAST-FUSION PRODUCTS

Introduction

The success of citrus breeding programs have been hampered by certain constraints such as sterility, sexual incompatibility, polyembryony, long juvenile period and the lack of information about the inheritance of important horticultural traits (Grosser and Gmitter 1990). The advances in biotechnology and molecular biology make available new techniques for enhancing the efficiency of citrus breeding programs. For instance, total or partial combination of the breeding progenitors can be feasible through somatic hybridization. The identity of new hybrids is confirmed using molecular analysis.

Citrus symmetric hybrids are allotetraploid ($2n=4x=36$), with intermediate phenotype of both parents, and high fertility (Ohgawara et al. 1991; Kobayashi et al. 1995). Somatic hybridization is a useful approach for overcoming the sexual incompatibilities between some citrus species (Saito et al. 1991; Kobayashi et al. 1991; Grosser and Gmitter 1990). In fact, somatic hybrids between citrus and sexually incompatible related genera have been generated (Grosser et al. 1996). Thus, it may be possible the transfer of interesting horticultural traits from citrus wide relatives into new citrus progenies. Intermediate degree of resistance to 'mal secco' (*Phoma tracheiphila*) disease was identified for 'Valencia' sweet orange + 'Femminello' lemon somatic hybrid (Tusa et al. 2000).

Citrus scion improvement programs worldwide have included interspecific and closely-related genera somatic hybridization as a complementary breeding technique aimed at developing breeding progenitors. Allotetraploid breeding parents are used in interploid hybridization to generate seedless triploid citrus and to introgress new genes in particular citrus genome (Grosser et al. 1998; Grosser et al. 2000).

Some somatic hybrids have been generated in the acid citrus fruit group. The 'Key' lime + 'Valencia' sweet orange somatic hybrid was attempted to transfer cold hardiness, resistance to lime anthracnose (*Gloeosporium limetticolum*) and tolerance to citrus tristeza virus from sweet orange to 'Key' lime and reduce seed number per fruit and increase fruit size in a 'Key' lime type fruit (Grosser et al. 1989).

A somatic hybrid containing 'Valencia' sweet orange and 'Femminello' lemon genomes was produced in order to improve tolerance to "mal secco" disease (*Phoma tracheiphila*) (Tusa et al. 1990). For similar purpose, somatic hybrids have been obtained using 'Hamlin' sweet orange and 'Milam' (a selection of *Citrus jambhiri*) as embryogenic protoplast donors and 'Femminello' as leaf protoplast donor (Tusa et al. 1992). Combinations of acid citrus protoplasts using *Citrus sudachi* and *Citrus aurantifolia* (Saito et al. 1991) and 'Murcott' tangor and 'Messina' lemon (Deng et al. 1995) have also been reported.

Protoplasts are sensitive entities whose regeneration is affected by several factors, for instance age and physiological conditions of protoplast donors, enzyme solution, nutrient medium, and plating density. Most of the citrus hybridization research by protoplast fusion is focused on developing interesting genome combinations, rather than evaluation of factors involved in the regeneration of protoplast fusion products and the

efficiency of tetraploid production. This study was performed to increase the acid citrus breeding parent germplasm by combining the lemon genome with other citrus species. It was also studied the regeneration of protoplast-fusion products and the efficiency of tetraploid production in relation to the initial protoplast nutrient media and the protoplast fusion technique.

Materials and Methods

Enzyme Digestion of Leaf Tissue

Budded trees or seedlings were grown under greenhouse conditions at low light intensity. Trees were periodically pruned and fertilized to induce new shoots. Fully expanded, but still young leaves were chosen. The procedures for protoplast isolation and fusion were previously described (Grosser and Gmitter 1990), with few modifications. Leaves were surface sterilized with 20% commercial bleach (sodium hypochlorite) and 2 drops/L Tween 20. After 15-20 min. leaves were rinsed three times with autoclaved-dionized water. If contamination could not be controlled with bleach, a quick immersion in 0.1N HCl before bleach treatment was applied. Fine cuts were made perpendicular to the midvein. The midvein was cut off and the leaf pieces (approximately 1 g) were incubated in a solution containing 8 mL enzyme solution (0.5% Macerace, 0.1% Pectolyase and 0.5% Cellulase, 0.7M Mannitol, 0.024M CaCl_2 , 6.15M MES and 0.92M NaH_2PO_4) and 3 mL 0.6M BH3 medium (Appendix C). To maximize the enzyme digestion, vacuum was applied for 20-30 min. The selected leaf protoplast donors were: *C. ichangensis*, *C. aurantium* cv. 'Chinotto', *C. medica* cv. 'Etrog', *C. micrantha* and the 'Lakeland' limequat hybrid.

Enzyme Digestion of Embryonic Cell Suspension

About 500 mg of tissue was collected from embryogenic cell suspension cultures that were initiated from ovule and sub-cultured every fifteen days in H-H EME medium (Appendix F), suggested by Grosser and Gmitter (1990). Tissue was placed into 60x15 mm Petri plate (Falcon, Lincoln Park, N.J.), subsequently the excess of nutrient medium was removed with a Pasteur pipette, then 1.50 mL of previously specified enzyme solution and 2.5 mL 0.7M BH3 (Appendix C) were added. The suspension was homogenized and the Petri plate sealed with Nescofilm® (Karlson Research Products Corp. S. Rosa, CA). 'Santa Teresa' and 'Lapithiotiki' lemons were used as cell-suspension protoplast donors in several somatic hybridization combinations, while 'Cavers' and 'Todo el año' were simply combined with 'Etrog' citron. The leaf and cell-suspension tissues were incubated on a rotatory shaker at 28 °C in dark for 16-18 hours.

Protoplast Purification

Thereafter, the suspensions were filtered using a 45- μ m stainless steel filter and protoplast pellets were recovered after centrifugation at 1000 rpm for 10 min. A gradient of CPW 25 sucrose and CPW 13 Mannitol (Appendix D) was used for protoplast purification. Protoplast pellets were resuspended in 5 mL sucrose, then 2 mL Mannitol was added softly to avoid the mixture of solutions. Protoplasts were isolated as a ring between solutions after centrifugation at 1000 rpm for 5 min. Protoplasts were pipetted out and rinsed in 0.6 M BH3. Leaf and cell-suspension protoplasts were mixed in 1:1 ratio after counting with a hemacytometer. Planting density was adjusted to 6×10^5 protoplast/mL.

Protoplast Fusion

Two drops of protoplast mixture were placed at the center of 60x 15 mm Petri plate, and then a drop of 40% (w/v) polyethylene glycol (PEG) 8000, was put in the center of the protoplast mixture followed by a second drop after adding the first drop to the set of plates. After 8 min. two drops of A and B solutions (Appendix E) in proportion 9:1 were placed in opposite sides of the mixture and incubated for 12 min. Then, three successive rinses using 0.6M BH3 medium were applied at 5 min. intervals to remove PEG and the A-B solution mixture. After the last rinse, 20 drops of culture medium were added. The cell suspension protoplast donors were 'Santa Teresa' and 'Lapithiotiki' lemons.

Regeneration of Protoplast Fusion Products

Protoplast-fusion products were cultured in three nutrient media: 0.6M BH3, 0.6M EME (Appendix A) and a mixture of both media in equal proportion. Cultures were fed with a mixture of 0.6 M BH3, 0.6M EME and 0.15M EME (Appendix A) in equal proportion after the second or third week of incubation. When solid micro-colonies developed, cultures were transferred to a sequence of media to induce embryogenesis. At transferring, a mixture of 0.6M BH3 and 0.15M EME (1:2 ratio) was added to the culture and the suspension was pipetted and transferred to solid 0.15 M EME. The media were in order of use: EME, 1500 EME (Appendix A) supplemented with maltose or sucrose, B+ (Appendix G) and DBA3 (Appendix H). Transferring to a fresh medium was performed monthly, and the medium type was selected according the somatic embryo development. All media and solutions were filter-sterilized using disposable 0.22 μ m-filter containers. Cultures were kept in plastic boxes under diffuse light until the moment they were transferred to solid medium, when continuous light was provided. The culture

room temperature was approximately 28°C. Four replications of 4 plates each were grown per medium for each protoplast combination. Mean number of embryo clusters regenerated per fusion plate in each medium was recorded. Embryos cluster was an embryogenic callus with many small embryos.

Somatic Hybrid Identification

Ploidy Analysis. The ploidy level and frequency of polyploids was determined by making a pool of embryo clusters to sample all or a maximum of 20 embryo clusters per Petri plate. The method applied for ploidy analysis is described in chapter 2. Polyploid embryos or embryo clusters were transferred to adequate medium to induce embryo development. Plantlets were grown on RMA to induce roots and plant growth, thereafter they were transplanted to soil.

Molecular analysis. Identity of putative somatic hybrids was confirmed utilizing RAPD (random amplified polymorphic DNA) primers. Genomic DNA was extracted from about 100 mg of young leaves using GenElute™ Plant Genomic DNA Miniprep Kit, Sigma, Inc. (St. Louis, MO). Leaf and embryogenic protoplast donors and two morphologically different regenerated polyploids were sampled. Amplification of RAPD markers was performed using 1-2 µL extracted DNA and 10-mer and 12-mer primers (Table 6.1). All primers were synthesized by Qiagen Operon (Alameda, CA). The PCR amplification program for 10-mer primers was 1. 94°C/5 min, 2. 94°C/1 min, 3. 35°C/1 min., 4. 72 °C/2min, 5. 40 cycles from 2-4 and 6. 72 °C/10 min. While 12-mer primers were amplified applying 1. 94°C/2 min. 2. 94°C/1 min. 3. 42°C/1 min. 4. 72 °C/2 min. 5. 34 cycles from 2-4 and 6. 72°C/10 min. The amplified products were electrophoretically separated in 1.4-1.8 % agarose gel that contained TAE buffer (Tris-Acetate-EDTA) and 1µL/100 mL ethidium bromide.

Evaluation of Two Protoplast Fusion Techniques

The previously described protocol for protoplast fusion was compared with a modified protocol routinely applied for citrus protoplast transformation (Fleming et al. 2000). This technique consists in carrying out the protoplast fusion in polystyrene culture tubes (17x100 mm), where 0.5 mL of adjusted protoplast mixture is added, and then 0.5 mL 40% PEG. After 15 min 0.5 mL A-B solution was added, following 15 min, 2 mL 0.6 M BH3 was added, followed by the addition of 4 more mL 0.6M BH3 after 15 min. Ten min. later, the mixture was centrifuge at 700 rpm for 10 min. The supernatant was removed, and one more rinse was applied using 4 mL BH3. After centrifugation (700 rpm for 10 min), protoplasts were resuspended in 2 mL of culture medium. Protoplast mixture was aliquotated into 5 Petri plates (60 x 15mm). Finally, the plates were sealed with Nescofilm (Karlhan Research Products Corporation, Santa Rosa CA). Culture conditions and experiment arrangement are described in the previous experiment. 'Lapithiotiki' lemon + 'Etrog' citron was the parental combination chosen for this experiment.

Results

Viability of protoplast fusion products that included 'Lapithiotiki' lemon decreased 24 hours after fusion. About half of plated protoplast survived reaching 57.49, 50.83 and 53.05% in 0.6BH3, 0.6EME and the mix medium, respectively. In general, after ten days cells began dividing to produce micro-colonies that could be distinguished after the second week of incubation. A month later, a high density of micro-colonies with solid appearance was observed. Average micro-colony size was approximately 1200 μ m. Development timing of high micro-colony density varied among genotype combinations, it varied between one and two months. Some green embryos were observed within a

week after the transfer to solid EME medium, other micro-colonies grew to develop embryogenic calli that differentiated into embryo clusters.

Regeneration of protoplast fusion products was inconsistent; considerable differences were observed within and among culture repetitions. Apparently, the initial medium did not affect remarkably the number of embryo clusters recovered per fusion plate. However, it was noticed that the number of embryo clusters was reduced in 'Santa Teresa' lemon + *C. micrantha* somatic hybridization, when 0.6M EME was the initial nutrient medium (Table 6.2). Similarly, 'Lapithiotiki' lemon + 'Lakeland' limequat (Table 6.3) developed more embryo clusters in all nutrient media except in 0.6M EME.

Regarding the ploidy of regenerated embryo clusters, variations of tetraploid ($2n=4x=36$) frequencies were observed among protoplast fusion combinations and media. Tetraploids were identified in all combinations including 'Santa Teresa' lemon (Table 6.2). Nonetheless, higher tetraploid frequencies were obtained from 'Lapithiotiki' (Table 6.3). In general, all combinations with 'Chinotto' sour orange showed the lowest tetraploid frequencies (Tables 6.2 and 6.3). Few hexaploids ($2n=6x=54$) were identified in some protoplast combinations that included 'Santa Teresa', with higher incidence in 'Lapithiotiki'. In most of the 'Lapithiotiki' lemon protoplast fusions, the percentages of recovered tetraploid were lower in the mixture BH3-EME medium (Tables 6.3 and 6.4).

Molecular analysis of 'Santa Teresa' tetraploid progenies showed that all screened plants were autotetraploids except a single plant from 'Santa Teresa' lemon + *C. ichangensis*, which was identified using the C64 primer (CCA GAT CCG AAT) (Figure 6.1). This suggests a low regeneration capacity of 'Santa Teresa' heterokaryonts. The

'Santa Teresa' lemon + *C. ichangensis* somatic hybrid has a large petiole wing that is a typical characteristic of *C. ichangensis* (Figure 6.2).

After screening several primers, allotetraploid somatic hybrid progenies were confirmed for all 'Lapithiotiki' lemon combinations. Figure 6.3 shows the results of RAPD-PCR analysis using the W-09 primer (GTG ACC GAG T) for 'Lapithiotiki' lemon + 'Lakeland' limequat hybrid and 'Lapithiotiki' lemon + 'Chinotto' sour orange progenies and the donor plants. The two tested plants from the 'Chinotto' sour orange somatic hybrid showed complementary bands from both donors, but they also have different polymorphisms. These plants are also phenotypically different; one of them has shorter internodes and smaller leaves resembling 'Chinotto' trees. Figure 6.4 shows leaf morphologies of somatic hybrid progenies obtained from 'Lapithiotiki' lemon as embryogenic cell suspension protoplast donor and four leaf protoplast donors. 'Lakeland' limequat and 'Lapithiotiki' lemon have similar leaf shapes; therefore minor differences can be noticed in the resulting somatic hybrids.

Embryogenesis was not affected by culture media when different protoplast fusion techniques were applied to fuse 'Lapithiotiki' lemon with 'Etrog' citron.. Fusion technique did not influence the regeneration of protoplast fusion products, but it certainly affected the amount of tetraploid and hexaploid embryo clusters regenerated (Table 6.4). More tetraploids were recovered when the fusion was performed in small culture tubes compared to results obtained in Petri plate. It was also noticed that the number of regenerated tetraploids was lower in the BH3-EME mixture than in the other media. A few hexaploids were regenerated in all media; however the hexaploids obtained in EME

and the BH3-EME mixture were only induced when the protoplast fusion was carried out in culture tubes.

Discussion

Lemon embryogenic cell-suspension derived protoplasts showed high embryogenic competence after interspecific protoplast fusions. The embryogenic competence of citrus protoplast obtained from ovule-derived callus was initially demonstrated by Vardi (1977). Furthermore, citrus plants regenerated from protoplast posse a high genetic stability (Kobayashi 1987). These characteristics make the somatic hybridization an appropriate technique for citrus genetic improvement. However, undifferentiated calli were still observed in most of the parental fusion combinations after four-five months of culturing on EME medium supplemented with sucrose or maltose. In some cultures the calli turned brown and died, whereas other calli grew without differentiation.

The initial nutrient medium affected the regeneration of protoplast fusion products in some combinations. The rich medium BH3 or the mixture of BH3 and EME induced embryogenesis more efficiently than EME, this suggests that organic compounds contributed to enhanced cell growth and development. The use of BH3 as initial citrus protoplast medium has been previously reported for citrus somatic hybridization of variable genome combinations (Grosser et al. 1990; Grosser et al. 1989) as well as the 1:1 mixture of BH3 and EME (Grosser et al. 1990; Grosser et al. 1992; Mourao et al. 1996).

On the contrary, EME medium is simple, containing the basic mineral composition, with sucrose and malt extract as supplements. 'Cook Eureka' lemon + '*C. aurantium*' cv. 'Chinotto' protoplasts only survived and developed in 0.6M EME medium, the two other media were not able to sustain live protoplasts and their development. However, tetraploids were not identified in over 100 tested plants. A medium similar to EME

supplemented with plant growth regulators, sucrose and mannitol has also been utilized for induction of somatic embryogenesis of citrus protoplasts (Kunitake et al. 1991) or without plant growth regulators for regeneration of protoplasts fusion products (Ling and Iwamasa 1994; Saito et al. 1991). Nevertheless, the effect of nutrient medium on protoplast regeneration was not discussed. Grosser and Gmitter (1990) recommended BH3 medium for recalcitrant genotypes.

Tetraploid and hexaploid embryos or embryo clusters were identified at early stage by flow cytometry. This technique reduced a considerable amount of sub-cultures allowing the further sub-culture of interesting polyploid material. The frequency of allotetraploid somatic hybrids varied according to the lemon protoplast donors. The initial culture medium mostly affected the tetraploid regeneration of 'Lapithiotiki' lemon protoplast fusions. 'Santa Teresa' protoplasts showed lower frequency of hybrids due to the autotetraploidy incidence. Regarding leaf protoplast donors, 'Etrog' showed the highest ability to fuse with other genotypes. On the other hand, 'Chinotto' sour orange leaf protoplast did not fuse in high frequency. This peculiarity might be related with protoplast size, which was smaller in 'Chinotto' sour orange compared to the other leaf protoplast donors.

Hexaploid hybrids were confirmed for 'Lapithiotiki' lemon + *C. ichangensis* and 'Lapithiotiki' lemon + 'Lakeland' limequat hybrid. Hexaploid hybrids have been previously reported for somatic hybridization between diploid citrus and a citrus relative. It was proposed that chromosome doubling occurred after fusion (Guo and Deng 1999). Difference in polymorphism between 'Lapithiotiki' somatic hybrids from the same combination might be associated with aneuploidy or DNA recombination. Genetic

variations in nuclear genomes have been confirmed among interspecific somatic hybrids from the same parental combination. These variations have been associated with mitotic recombination (Binsfeld and Schnabl 2002) and reduction in chromosome number (Liu et al. 1995; Buiteveld et al. 1998). In citrus, variation or recombination was observed among some wide somatic hybrids (Guo et al. 1998).

Currently, phenotypic differences of the new lemon somatic hybrids are basically determined by leaf shape and size, thorn and the inter-node length. All of them are very vigorous, lower vigor was noticed in the hexaploid trees.

Larger volume of protoplast mixture along with longer protoplast exposure to higher PEG volume definitely improved protoplast fusion. On the other hand, fusogenic solutions are better removed not only because of the larger amount of medium used for rinsing, but also because of the use of centrifugation to precipitate protoplasts. A notably higher frequency of tetraploids and the ease to accomplish protoplast fusion in culture tube made the protoplast fusion more efficient, reducing the high variability often observed when protoplast fusion is carried out in individual plates.

Novel lemon allotetraploid hybrids were generated that combine the genome of elite lemon varieties, such as 'Laptithiotiki', 'St. Teresa', 'Todo el año' and 'Cavers' with other citrus species that carry interesting horticultural traits, for instance small tree size ('Chinotto' sour orange), cold resistance (*C. ichangensis*), canker resistance ('Lakeland' limequat hybrid, 'Etrog' citron) and "mal secco" ('Chinotto' sour orange). Efficiency of allotetraploid somatic hybrid regeneration was enhanced by modifying protoplast fusion protocol in protoplast, medium and solution volumes and incubation time as well as the substitution of Petri dishes by culture tubes.

Table 6.1. Mean of embryo clusters and frequency of polyploids regenerated from 'Santa Teresa' lemon protoplast fused with several leaf donors and initially grown on different nutrient media.

Cell Suspension Donor Santa Teresa Lemon		Embryo Cluster	Polyploid Cluster (%)	
Leaf Donors	Medium	Total Number	4X	6X
<i>C. micrantha</i>	BH3	58.33±48.97	4.76±4.76	0
	EME	26.35±20.40	11.53±4.45	1.05±1.05
	BH3-EME	75.31±38.62	9.21±2.87	0
<i>C. aurantium</i> cv. 'Chinotto'	BH3	49.63±20.48	8.61±5.39	0
	EME	31.75±11.63	0	0
	BH3-EME	37.47±29.16	0.77±0.77	0
<i>C. ichangensis</i>	BH3	37.00±19.07	7.00±4.35	0
	EME	29.34±15.25	5.00±3.42	0
	BH3-EME	37.51±12.53	17.92±6.53	0
'Lakeland' limequat	BH3-EME	----	25	0

Table 6.2. Mean of embryo clusters and frequency of polyploids regenerated from 'Lapithiotiki' lemon protoplast fused with different leaf donors and initially grown on different nutrient media

Cell Suspension Donor Lapithiotiki Lemon		Embryo Clusters /Petri plate	Polyploid Embryo Cluster (%)	
Leaf Parents	Medium		4X	6X
Lakeland limequat	BH3	60.39±32.55	42.64±15.09	10.60±7.23
	EME	27.00±14.04	29.4±15.704	0
	BH3-EME	71.47±38.62	11.34±2.33	0.30±0.30
<i>C. aurantium</i> cv. Chinotto'	BH3	11.05±4.95	10.00±10.00	0
	EME	28.40±9.00	5.77±3.68	0
	BH3-EME	28.63±8.46	1.59±1.59	0
<i>C. ichangensis</i>	BH3	28.43±8.15	11.64±5.82	0
	EME	32.88±17.48	19.79±15.36	0
	BH3-EME	45.46±10.82	16.89±6.05	1.75±1.32

Table 6.3. Effect of protoplast fusion techniques on protoplast regeneration and polyploid frequency of 'Lapithiotiki' lemon + 'Etrog' citron..

Medium	Fusion Technique	Embryo cluster	Tetraploid	Hexaploid
0.6M BH3	PEG-Plate	25.60±3.92	65.86±18.47	6.07±5.00
	PEG-Tube	25.51±1.02	90.00±5.77	5.00±5.00
0.6M EME	PEG-Plate	18.75±8.16	73.15±13.97	0
	PEG-Tube	25.15±2.73	96.25±2.39	1.25±1.25
0.6M BH3-EME	PEG-Plate	30.00±3.74	52.09±17.06	0
	PEG-Tube	21.99±3.42	78.61±11.00	2.50±2.50

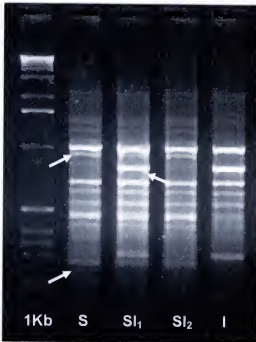


Figure 6.1. Amplified DNA banding patterns using the random primers C64. S='Santa Teresa'; I= *C. ichangensis*; 1-2 = tetraploids individuals. Arrows indicate complementary bands

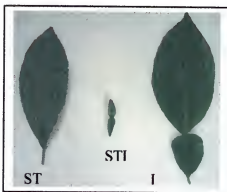


Figure 6.2. Leaf morphology of 'Santa Teresa' lemon + *C. ichangensis* somatic hybrid. ST= Santa Teresa; STI= Santa Teresa + *C. ichangensis*; I= *C. ichangensis*

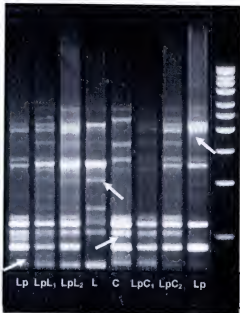


Figure 6.3. Amplified DNA banding pattern using W-09 primer. Lp= 'Lapithiotiki'; L= 'Lakeland' limequat; C= 'Chinotto' sour orange; 1-2= two different tetraploids.



Figure 6.4. Leaf morphology of 'Lapithiotiki' lemon + 'Lakeland' limequat and 'Lapithiotiki' lemon + 'Chinotto' sour orange somatic hybrids and their progenitors.

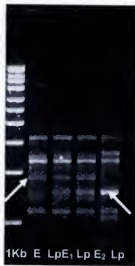


Figure 6.5. Amplified DNA banding pattern using U-18 primer. Lp= 'Lapithiotiki'; E='Etrog' citron; 1-2= two different tetraploids.

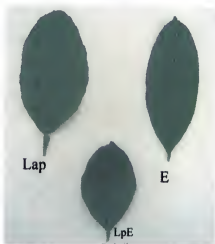


Figure 6.6. Leaf morphology of 'Lapithiotiki' lemon + 'Etrog' citron somatic hybrids and their progenitors. Lp= 'Lapithiotiki'; E='Etrog' citron; LpE + Lapithiotiki + Etrog.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The success of interploid hybridization was influenced by the ploidy level of the seed parents. Diploid trees were able to set more fruit with larger seed numbers compared to tetraploid seed progenitors. Most of the tetraploid x diploid crosses failed to set fruit. 'Hamlin' sweet orange + 'Femminello' lemon was the only seed progenitor that had fertile crosses, however the number of generated triploids was very low. The outcome from tetraploid x diploid crosses was totally unexpected, since mono- or partially monoembryonic autotetraploids have been recommended as citrus seed parents (Soost and Cameron 1969; Cameron and Burnett 1978). Citrus embryos are larger when the endosperm is pentaploid, although seeds are smaller in relation to seed with normal ploidy (Esen and Soost 1973). The high number of nucellar embryos obtained in the viable tetraploid-x diploid crosses made the hybrid recovery more difficult.

Lemons are seedier than the lime seed progenitors which is favorable for hybrid production. Parental genotype combination affected the frequency of triploid hybrids, with a tendency to generate more hybrids when 'Hamlin' sweet orange + 'Femminello' lemon was the pollen progenitor. Despite the low sexual compatibility of 'Valencia' sweet orange + 'Femminello' lemon with the selected seed progenitors, it was possible to produce some triploids.

Citron has been reported as a progenitor of several citrus species, including some of the diploid breeding progenitos used in this study, therefore it may contribute to interesting progenies in combination with the somatic hybrids. Progenies from

autotetraploid lemon and acid lime progenitors have potential to produce fruit with high acid content considering the previous report that indicated that progenies with high acid percentage were obtained from tetraploid lemon progenitors (Soost and Cameron 1969). The genomic combinations of generated triploid progenies should be valuable for the genetic improvement of the currently grown acid citrus fruit cultivars.

In vitro germination of acid citrus fruit embryos was mainly influenced by genotype, embryo developmental stage and nutrient medium. Overall, germination of globular, heart-shaped and early cotyledonary embryos was induced at a higher rate by Gamborg's B5 medium, whereas immature cotyledon embryos germinated well on either Gamborg's B5 or EME. Immature cotyledonary embryos from 'Limonero Fino 49' x HF showed the lowest germination rate with a very low number of recovered plants. These weak embryos were also affected by sucrose concentration. Recovery of normal seedlings from younger embryos was very low in spite of the good germination rate in the 2001 experiments.

Plant recovery from small embryos was higher for 'Todo el Año' progenies in the 2002 experiments. Embryos were grown in germination medium for two weeks. Growing media did not affect significantly the recovery of active growing seedlings. Considering the ploidy of germinated embryos that did not develop into active growing seedling as well as that of the recovered seedling, the percentage of normal diploid seedling was significantly higher than the percentage of normal triploid from 'Todo el Año' progenies, but no differences were found for 'Lisbon' seedlings.

The effect of two nutrient media and three concentrations of GA and sucrose were evaluated on germination of aborted mandarin triploids embryos. The embryo

germination rate was higher on Gamborg's B5 similar to small embryos of acid citrus fruit. The addition of GA promoted germination, and the sucrose effect was influenced by GA concentration. Germination rate was low when GA was lacking at any concentration of sucrose. Germination improved with the increases of GA in all sucrose concentrations, nonetheless the differences between 4.54 and 9.09 μM GA at any level of sucrose were not significant.

Nutrient media did not affect considerably the percentage of tetraploid hybrid regenerated. In some parental combinations, EME medium induced lower percentage of embryo clusters compared to the other media. Molecular analysis based on Random Amplified Polymorphic DNA confirmed the identity of regenerated hybrids. The success of somatic hybrid generation was hampered by the high incidence of autotetraploid in all parental combinations that included 'Santa Teresa' lemon. 'Santa Teresa' lemon + *C. ichangensis* was the only somatic hybrid confirmed. Somatic hybrids were identified in all tetraploid progenies from 'Lapithiotiki' lemon except when *C. ichangensis* was the leaf protoplast donor. Hybrids were identified from 'Lakeland' limequat hybrid, 'Etrog' citron, and *C. aurantium* cv. 'Chinotto' as leaf parents. These new tetraploid progenitors will increase the genetic variability of the current available germplasm for genetic improvement of acid citrus fruit cultivars.. They have potential for cold hardiness, reduced vigor, and Asiatic citrus canker resistance.

Screening for Asiatic canker resistance using a stomatal inoculation technique under greenhouse conditions showed through repetitive experiments that the triploid hybrids from 'Lakeland' limequat x 'Key' lime + 'Valencia' orange and 'Lakeland' limequat x 'Hamlin' orange + 'Femminello' lemon exhibited potentially useful levels of

resistance. This research indicates that canker resistance genes from kumquat can be readily moved into acid-fruit hybrids by conventional breeding.

APPENDIX A
COMPOSITION OF THE EME MEDIUM

Component	mg/L
NH ₄ NO ₃	1,650
KNO ₃	1,900
KH ₂ PO ₄	170
MgSO ₄ .7H ₂ O	370
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O	27.80
MnSO ₄ .H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
H ₃ BO ₃	6.20
KI	0.63
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Thiamine. HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5

50 g/L sucrose was added for 0.146 M EME and 205.38 g/L sucrose for 0.6 M EME. For 1500 EME malt extract was added at 1500 mg/L and sucrose at 50 g/L. Solid medium contains 8 g/L agar.

APPENDIX B
COMPOSITION OF RMA MEDIUM

Component	mg/L
NH ₄ NO ₃	825
KNO ₃	950
KH ₂ PO ₄	85
MgSO ₄ .7H ₂ O	185
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O (EDTA)	27.80
MnSO ₄ .H ₂ O	11.15
ZnSO ₄ .7H ₂ O	4.30
H ₃ BO ₃	3.10
KI	0.42
Na ₂ MoO ₄ .2H ₂ O	0.13
CuSO ₄ .5H ₂ O	0.013
CoCl ₂ .6H ₂ O	0.013
Thiamine. HCl	5
Pyridoxine.HCl	5
Nicotinic acid	0.50
Naphtalenacetic acid	0.020
Activated charcoal	500
Sucrose	25,000
Agar	8,000

APPENDIX C
COMPOSITION OF 0.6M BH3 NUTRIENT MEDIUM

Component	mg/L
KH ₂ PO ₄	170
MgSO ₄ .7H ₂ O	370
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O	27.80
MnSO ₄ .H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
H ₃ BO ₃	6.20
KCl	1,500
KI	0.63
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Glutamine	3,100
Thiamine. HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Casein hydrolysate	250
Nicotinic acid	1
Mannitol	81,990
Sucrose	51,350 (85,560 for 0.7 M)
Coconut water	20 mL
Fructose	250
Ribose	250
Xylose	250
Mannose	250
Rhamanose	250
Cellobiose	250
Galactose	250
Glucose	250
Sodium pyruvate	20
Citric acid	40
Malic acid	40
Fumaric acid	40
Vitamin B ₁₂	0.02
Calcium pantothenate	1
Ascorbic acid	2
Choline chloride	1
p-aminobenzoic acid	0.02
Folic acid	0.40
Riboflavin	0.20
Biotin	0.01
Vitamin a (retinol)	0.01
Vitamin D ₃ (cholecalciferol)	0.01

APPENDIX D
COMPOSITION OF SUCROSE AND MANNITOL SOLUTION GRADIENT

CPW SALTS	
	mg/ mL
MgSO ₄ . 7H ₂ O	250
KNO ₃	100
KH ₂ PO ₄	27.20
KI	0.16
CuSO ₄ .5H ₂ O	0.00025
CaCl ₂ .2H ₂ O	150

For CPW-25 Sucrose, 25 g/100 mL sucrose was added and for CPW-13 Mannitol 13 g/100 mL mannitol was added. pH of both solutions was 5.8.

APPENDIX E
COMPOSITION OF A AND B SOLUTIONS

Solution A		Solution B	
Component	g/100mL	Component	g/100mL
Glucose (0.4M)	7.20	Glycine (0.3 M)	2.2
CaCl ₂ (66 mM)	0.97		
DMSO	10 mL		
pH 6.0		pH 10.5	

APPENDIX F
COMPOSITION OF H-H EME MEDIUM

Component	mg/L
NH ₄ NO ₃	825
KNO ₃	950
KH ₂ PO ₄	170
MgSO ₄ .7H ₂ O	370
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O	27.80
MnSO ₄ .H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
H ₃ BO ₃	6.20
KI	0.63
KCl	750
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Thiamine. HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5
Glutamine	1,550
Sucrose	35,000

APPENDIX G
COMPOSITION OF B+ MEDIUM

Component	mg/L
NH ₄ NO ₃	1,650
KNO ₃	1,900
KH ₂ PO ₄	170
MgSO ₄ .7H ₂ O	370
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O	27.80
MnSO ₄ .H ₂ O	22.30
ZnSO ₄ .7H ₂ O	8.60
H ₃ BO ₃	6.20
KI	0.63
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Thiamine. HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5
Coconut water	20 mL
Coumarin	14.60
NAA	0.02
GA	1.0
Sucrose	25,000
Agar	8,000

APPENDIX H
COMPOSITION OF DBA3 MEDIUM

Component	mg/L
NH ₄ NO ₃	1485
KNO ₃	1710
KH ₂ PO ₄	153
MgSO ₄ .7H ₂ O	333
CaCl ₂ .2H ₂ O	440
Na ₂ EDTA	37.30
FeSO ₄ .7H ₂ O	27.80
MnSO ₄ .H ₂ O	21.40
ZnSO ₄ .7H ₂ O	7.7
H ₃ BO ₃	5.58
KI	0.567
Na ₂ MoO ₄ .2H ₂ O	0.225
CuSO ₄ .5H ₂ O	0.0225
CoCl ₂ .6H ₂ O	0.0225
Thiamine. HCl	9
Pyridoxine.HCl	9
Myo-inositol	90
Nicotinic acid	4.5
Coconut water	20 mL
Malt extract	1,500
2,4 D	0.01
BAP	3
Sucrose	25,000
Agar	8,000

LIST OF REFERENCES

- Alonzo, G., S. Fatta del Bosco, E. Palazzolo, F. Saiano, and N. Tusa. 2000. Citrus somatic hybrid essential oil. *Flavour and Fragrance Journal* 15:258-262.
- Bajaj, Y. P. S. 1994. Somatic hybridization a rich source of genetic variability. IN: Y. P. S. Bajaj (ed). *Biotechnology in agriculture and forestry: Somatic hybridization in crop improvement I* 27:3-32. Springer-Verlag Berlin, Heidelberg.
- Barrett, H.C. 1985. Hybridization of *Citrus* and related genera. *Fruit Varieties Journal* 39:11-16.
- Barrett, H.C. 1992. An autotetraploid of the key lime, *Citrus aurantifolia*. *Fruit Varieties Journal* 46:166-170.
- Binsfeld, P. C., and H. Schnabl. 2002. Molecular and cytogenetic constitution of plant obtained via two different somatic hybridization methods. *Plant Cell Reports* 21:58-62.
- Bodanese-Zanettini M. H., M. S. Lauxen, S. N. C. Richter, S. Cavalli-Molina, C. E. Lange P.I. Wang, C.Y. Hu. 1996. Wide hybridization between Brazilian soybean cultivars and wild perennial relatives. *Theoretical and Applied Genetics* 93:703-709.
- Buiteveld, J., Y. Suo, M. M. van Lookeren Campagne, J. Creemers Molenaar. 1998. Production and characterization of somatic hybrid plants between leek (*Allium ampeloprasum* L.) and onion (*Allium cepa* L.). *Theoretical and Applied Genetics* 96:765-775.
- Cameron, J. W., and H. B. Frost. 1969. Genetics, breeding, and nucellar embryony. IN: W. Reuther, L. D. Bachelor, H. J. Webber. *The citrus industry*. Vol. II:325-370. University of California, Berkeley.
- Cameron, J. W., and R. H. Burnett. 1978. Use of sexual tetraploid seed parents for production of triploid *Citrus* hybrids. *Horticultural Science* 13:167-169.
- Cameron, J. W., and R. K. Soost. 1969. Characters of new populations of *Citrus* polyploids and the relation between tetraploidy in the pollen parent and hybrid tetraploid progeny. *Proceedings of the First International Citrus Symposium* 1:199-205.

- Carimi, F., F. De Pasquale, and A. M. Puglia. 1998. *In vitro* rescue of zygotic embryos of sour orange, *Citrus aurantium* L., and their detection based on RFLP analysis. *Plant Breeding* 117: 261-266.
- Carputo, D., L. Monti, J. E. Werner, and L. Frusciante. 1999. Uses and usefulness of endosperm balance number. *Theoretical and Applied Genetics* 98:478-484.
- Chandler, J., and B. Beard. 1983. Embryo culture of *Helianthus* hybrids. *Crop Science* 23:1004-1007.
- Chaparro, J. X. and W. B. Sherman. 1994. Culture date and germination procedure affects success of nectarine ovule and embryo culture. *Fruit Varieties Journal*. 48:173-175.
- Civerolo, E. L. 1985. Citrus canker disease: The bacterium *Xanthomonas campestris* pv. citri. IN: Timmer, L. W (ed.). Citrus canker: an international perspective. Symposium 2-7.
- Collins, G. B. and J. W. Grosser. 1984. Culture of embryos. IN: Vasil, I. K. (ed.). Cell culture and somatic cell genetics of plants. Vol. 1:241-253. Academic Press, Inc., New York
- Continella G., G. La Malfa, and D. Romano. 1992. The utilization of citrus as ornamental plants in Italy. *Proceedings International Society of Citriculture* 1:232-234.
- Deng, X. X., H. L. Yi, F. Li, and W.W. Guo. 1996. Triploid plants regenerated from crossing diploid pummelo and tangerine with allotetraploid somatic hybrid of citrus. *Proceedings of the International Society of Citriculture* 1:189-192.
- Dienelt, M. M. and R. H. Lawson. 1989. Histology of *Xanthomonas campestris* pv. citri from Florida and Mexico in wound-inoculated detached leaves of Citrus aurantifolia: transmission Electron microscopy. *Phytopathology* 79:336-348.
- Din Khan I. and M. K. Hingorani. 1970. Strain studies in *Xanthomonas citri* (Hasse) Dowson. *Journal of Horticultural Sciences* 45:15-17.
- Emershad, R. L. and D. W. Ramming. 1994. Effect of media on embryo enlargement, germination and plant development in early-ripening genotypes of *Prunus* grown in vitro. *Plant Cell Tissue and Organ Culture* 37:55-59.
- Esen, A., and R. K. Soost. 1971. Unexpected triploid in *Citrus* : their origin, identification and possible use. *Journal of Heredity* 62:329-333.
- Esen, A., and R. K. Soost. 1972a. Aneuploidy in Citrus. *American Journal of Botany* 59:473-477.

- Esen, A., and R. K. Soost. 1972b. Tetraploid progenies from 2X x 4X crosses of citrus and their origin. *Journal of the American Society for Horticultural Science* 97:410-414.
- Esen, A., and R. K. Soost. 1973. Seed development in citrus with special reference to 2X x 4X crosses. *American Journal of Botany* 60: 448-462.
- FAO-AGRIS. Statistical Databases. 2002.
<http://apps.fao.org/page/collections?subset=agriculture>, Enero 15, 2003.
- Fatta del Bosco, S., E. Palazzolo, M.T. Scarano, M.A. Germana, and N. Tusa. 1998. Comparison between essential oil yield and constituents of an allotetraploid somatic hybrid of *Citrus* and its parents. *Advances in Horticultural Science* 12:72-77.
- Fleming, G. H., O. Olivares Fuster, S. Fatta del Bosco, and J. W. Grosser. 2000. An alternative method for the genetic transformation of sweet orange. *In vitro Cellular and Developmental Biology* 36:450-455.
- Florida Department of Agriculture. 2002a. Citrus summary. Florida agricultural Statistics Service.
- Florida Department of Agriculture. Tropical fruit, acres and trees. 2002b.
<http://www.nass.usda.gov/fl> February 19, 2003.
- Garnier, M., L.Zreik and J.M. Bové. 1991. Witches'-broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Disease* 75:546-551.
- Garnsey S. M. and T. Miyakawa. 1988. Tristeza. IN: Whiteside J. O. S. M. Garnesy and L. W. Timmer (eds). *Compendium of citrus diseases* 48-51. APS St. Paul Mn.
- Gazea, F., I. Calvarano, M. Calvarano, G. Russo, and A. Starrantino. 1996. Essential oil composition of a triploid lemon hybrid. *Proceedings of the International Society of Citriculture* 1:331-333.
- Gottwald, T. R., J. H. Graham, E. L. Civerolo, H. C. Barrett and C. J. Hrn. 1993. Differential host range reaction of citrus and citrus relatives to citrus canker and citrus bacterial spot determined by leaf mesophyll susceptibility. *Plant Disease* 77:1004-1009.
- Gottwald, T. R., J. H. Graham, Schubert, T. S. 2002. Citrus canker: the pathogen and its impact. On line Plant Health Progerss doi:10:1094/PHP-2002-0812-01-RV.
- Gottwald, T. R., J. H. Graham. 1992. A device for precise and nondisruptive stomatal inoculation of leaf tissue with bacterial pathogens. *Phytopathology* 82:930-935.

- Graham, J. H., T. R. Gottwald and D. Fardelmann. 1990. Cultivar-specific interactions for strains of *Xanthomonas campestris* from Florida that cause citrus canker and citrus bacterial spot. *Plant Disease* 74:753-756.
- Graham, J. H., T. R. Gottwald, T. D. Riley and D. Achor. 1992. Penetration through leaf stomata and growth of strains and *Xanthomonas campestris* in citrus cultivars varying in susceptibility to bacterial diseases. *Phytopathology* 82:1319-1325.
- Gray, D. J., and L. A. Hanger. 1993. Effect of ovule maturity on recovery of zygotic embryos and embryogenic cultures from muscadine grape. *HortScience* 28:227.
- Grosser, J. W., and F. G. Gmitter, Jr. 1990. Protoplast fusion and citrus improvement. *Plant Breeding Review* 8:339-374.
- Grosser, J. W., F. A. A. Mourao-Fo, F. G. Gmitter Jr., E. S. Louzada, J. Jiang, A. Quiros, C. Cabasson, L. Schell, and J. L. Chandler. 1996. Allotetraploid hybrids between citrus and seven related genera produced by somatic hybridization. *Theoretical and Applied Genetics* 92:577-582.
- Grosser, J. W., F. G. Gmitter Jr., E. S. Louzada, and J. L. Chandler. 1992a. Production of somatic hybrid and autotetraploid breeding parents for seedless citrus development. *HortScience* 27:1125-1127.
- Grosser, J. W., F. G. Gmitter Jr., F. Sesto, X. X. Deng, and J. L. Chandler. 1992b. Six new somatic hybrids and their potential for cultivar improvement. *Journal of the American Society for Horticultural Science* 117:169-173.
- Grosser, J. W., G. A. Moore, and F. G. Gmitter Jr. 1989. Interspecific somatic hybrid plants from the fusion of 'Key' lime (*Citrus aurantifolia*) with 'Valencia' sweet orange (*Citrus sinensis*) protoplasts. *Scientia Horticulturae* 39:23-29.
- Grosser, J. W., J. Jiang, F. A. A. Mourao-Fo, E. S. Louzada, K. Baergen, J. L. Chandler and F. G. Gmitter Jr. 1998. Somatic hybridization, an integral component of citrus cultivar improvement: I. Scion improvement. *Horticultural Science* 33:1057-1059.
- Grosser, J. W., P. Ollitrault, and O. Olivares-Fuster. 2000. Somatic hybridization in citrus: an effective tool to facilitate variety improvement. *In vitro Cellular and Developmental Biology* 36:434-449.
- Gulsen, O., and M. L. Roose. 2001. Chloroplast and nuclear genome analysis of the parentage of lemons. *Journal of the American Society for Horticultural Science* 126:210-215.
- Guo, W. W., and X. X. Deng. 1999. Intertribal hexaploid somatic hybrid plants regeneration from electrofusion between diploids of *Citrus sinensis* and its sexually incompatible relative, *Clausena lansium*. *Theoretical and Applied Genetics* 98:581-585.

- Guo, W. W., and X. X. Deng. 2001. Wide somatic hybrids of *Citrus* with its related genera and their potential in genetic improvement. IN: S. M. Jain, B. S. Ahloowalia, G. S. Khush, H. Zhu Li. Genetics- better life for all. Selected Proceedings of the 18th International Congress of Genetics, Beijing, China, 1998. Euphytica 118:175-183.
- Hearn, C. J. 1984. Development of seedless orange and grapefruit cultivars through seed irradiation. Journal of the American Society for Horticultural Science. 109:270-273.
- Hearn, C. J. 1985. Citrus scion improvement program. Fruit varieties Journal. 39: 35-37.
- Hensz, R. A. 1977. Mutation breeding and the development of the 'Star Ruby' grapefruit. Proceedings of the International Society of Citriculture 2:582-585.
- Hirsch, A. M., R. Testolin, S. Brown, J. Chat, D. Fortune, J. M. Bureau, and D. De Nay. 2001. Embryo rescue from interspecific crosses in the genus *Actinidia* (kiwifruit). Plant Cell Reports 20:508-516.
- Johnston, S. A., T. P. M. den Nijs, S. J. Peloquin, and R. E. Hanneman. 1980. The significance of genic balance to endosperm development in interspecific crosses. Theoretical and Applied Genetics 57:5-9.
- Kale K. B., S. O. Kolte, and N. L. Peshney. 1994. Economics of chemical control of citrus canker caused by *Xanthomonas campestris* pv. *citri* under field conditions. Indian Phytopathology 47:253-255. Abstract.
- Kaneyoshi, J., T. Kanou, Y. Kuwata, A. Hirao, S. Nakatani, and S. Kobayashi. 1997. Breeding of triploid citrus cultivars I. Production of triploids from satsuma mandarin (*Citrus unshiu* Marc.) X tetraploid ponkan mandarin (*Citrus reticulata* Blanco) crosses. Journal of the Japanese Society for Horticultural Sciences 66:9-14. Abstract.
- Kapur S., S. S. Cheema and S. P. Kapur. 1997. Efficacy of different chemotherapeutants for control of citrus canker, Plant Disease 12:159-161.
- Karssen, C. M., S. Zagorski, Kepczynski and S. P.C. Groot. 1989. Key role for endogenous gibberellins in the control of seed germination. Annals of Botany 63:71-80.
- Khan, I. A., and M. L. Roose. 1988. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. Journal of the American Society for Horticultural Science 113:105-110.
- Khan, I. A., M. J. Jaskani, and S. N. Haider Ali. 1996. Interploid hybridization for the improvement of Kinnow mandarin. Proceedings of the International Society of Citriculture 137-140.

- Knorr, L. C. 1958. Finding the best lemon in Florida – a report progress. Proceedings of the Florida State Horticultural Society 71:124-146.
- Kobayashi, S. 1987. Uniformity of plants regenerated from orange (*Citrus sinensis* Osb.) protoplast. Theoretical and Applied Genetics 74:10-14.
- Kobayashi, S., I. Oiyama, K. Yoshinaga, T. Ohgawara, and S. Ishii. 1991. Fertility in an intergeneric somatic hybrid plant of Rutaceae. HortScience 26:207.
- Kobayashi, S., T. Ohgawara, W. Saito, Y. Nakamura, and J. Shimizu. 1995. Fruit characteristics and pollen fertility of citrus somatic hybrids. Journal of the Japanese Society of Horticultural Science 64:283-289. Abstract.
- Koizumi, M. 1981. Resistance of citrus plants to bacterial canker disease: a review. Proceedings of the International Society of Citriculture Vol. 1 402-405.
- Koizumi, M. 1985. Citrus canker: the world situation. IN: Timmer, L. W. (ed.). Symposium of Citrus Canker: an international perspective 2-7.
- Koltunow, A. M., A. Vivian Smith and S. R. Sykes. 2000. Molecular and conventional breeding strategies for seedless citrus. Proceedings of the First International Symposium on Citrus biotechnology. Acta Horticulturae 535:189-174.
- Kunitake, H., H. Kagami, and M. Masahiro. 1991. Somatic embryogenesis and plant regeneration from protoplast of 'Satsuma' mandarin (*Citrus unshiu* Marc.). Scientia Horticulturae 47:27-33.
- Leite, R. P. and S. K. Mohan. 1984. Evaluation of citrus cultivars for resistance to canker caused by *Xanthomonas campestris* pv. *citri* (H. asse) Dye in the state of Parana, Brazil. Proceedings of the International Society of Citriculture Vol. 1 35-38.
- Ling, J. T., and M. Iwamasa. 1994. Somatic hybridization between *Citrus reticulata* and *Citropsis gabunensis* through electrofusion. Plant Cell Reports 13:493-497.
- Liu, J. H., C. Dixelius, I. Eriksson and K. Glimelius. 1995. Brassica napus(+) B. tounefortii, a somatic hybrid containing traits of agronomic importance for rapeseed breeding. Plant Science. 109:75-86.
- Lopez M. M. and L. Navarro. 1981. A new in vitro inoculation method for citrus canker diagnosis. Proceedings of the International Society of Citriculture Vol 1:399-402.
- Lyman Ott, R. 1993. An introduction to statistical methods and data analysis. Duxbury Press. Belmont, California. 1183 pp.
- Magdalita, P. M., S. W. Adkins, L. D. Godwin, and R.A. Drew. 1996. An improvement embryo-rescue protocol for a *Carica* interspecific hybrid. Australian Journal of Botany 44:343-353.

- Medina Urrutia, V., and M. Buenrostro Nava. 1995. Effect of Paclobutrazol on vegetative growth, flowering, fruit size and yield in Mexican lime (*Citrus aurantifolia*) trees. Proceedings of the Florida State Horticultural Society 108:361-364.
- Morton, J. F. 1987. Fruits of warm climates. Media, Incorporated Greensboro, N.C. 505 pp.
- Mourao, F.A., F.G. Gmitter and J.W. Grosser. 1996. New tetraploid breeding parents for triploid seedless citrus cultivar development. Fruit Varieties Journal 50:76-80.
- Murashige, T., and D. P. H. Tucker. 1969. Growth factor requirements of citrus tissue culture. Proceedings of the First International Citrus Symposium 3:1155-1161.
- Murashige, T., and Skoog, F. M. 1962. A Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarum. 15:473-497.
- Nerderwieser, J. G., A. A. van de Venter and P. J. Robbertse. 1990. Embryo rescue in *Ornithogalum*. HortScience 25:565-566.
- Nicolosi, E., Z. N. Deng, A. Gentile, S. La Malfa, G. Continella, and E. Tribulato. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics 100:1155-1166.
- Ohgawara, T, Kobayashi, S, Ishii, S., K. Yoshinaga, and I. Oiyama. 1991. Fertile fruit trees obtained by somatic hybridization: navel orange (*Citrus sinensis*) and Troyer citrange (*Citrus sinensis* x *Poncirus trifoliata*). Theoretical and Applied Genetics 81:141-143.
- Oiyama I., and S. Kobayashi. 1990. Polyembryony in undeveloped monoembryonic diploid seeds crossed with citrus tetraploid. HortScience 25:1276-1277.
- Oiyama I., and S. Kobayashi. 1991. Citrus pentaploids from small seeds of diploid x diploid crosses. HortScience 26:292-293.
- Pinto, A. C. Q., S. M. Dethier Rogers, and D. H. Byrne. 1994. Growth of immature Peach embryos in response to media, ovule support method, and ovule perforation. HortScience 29:1081-1083.
- Prasad, M. B. N. V., R. Singh, A. Rekha, R. Chand. 1997. Evaluation of lemon cultivars and acid lime x lemon hybrids for resistance to *Xanthomonas axonopodis* pv. citri. Scientia Horticulturae 71: 267-272.
- Qi, G. M.; C. Y Pan, X. N Tang, and L. Y. Yang. 1998. Cultural techniques for seedless grape hybrid ovules. Journal of Fruit Science 4:364-367.
- Raghuvanshi, S. S. 1969. Cytological evidence bearing on evolution in *Citrus*. Proceedings of the First International Citrus Symposium 1:207-214.

- Ramming, D. W. 1985. In ovulo embryo culture of early-maturing *Prunus*. *HortScience* 20: 419-120.
- Ramming, D. W. 1990. The use of embryo culture in fruit breeding. *HortScience* 25:393-398.
- Rangan, T. S., T. Murashige, and W. P. Bitters. 1969. In vitro studies of zygotic and nucellar embryogenesis in citrus. *Proceedings of the International Society of Citriculture* 1:225-229.
- Reddy, M. R. S. 1997. Sources of resistance to bacterial canker in citrus. *Journal of Mycology and Plant Pathology* 27:80-81.
- Rietsema, J. S. Satina, and A.F. Blakeslee. 1953. The effect of sucrose on the growth of *Datura stramonium* embryos *in vitro*. *American journal of Botany* 46:538-545.
- Ruberto, G., A. Renda, M. Piattelli, P. Rapisarda, and A. Starrantino. 1997. Essential oil of two new pigmented citrus hybrids *Citrus clementina* x *Citrus sinensis*. *Journal of Agricultural Food and Chemistry* 45:467-471.
- Saito, W., T.Ohgawara, J. Shimizu, and S. Ishii. 1991. Acid citrus somatic hybrids between *Sudachi* (*Citrus sudachi* Hort. ex Shirai) and lime (*C. aurantifolia* Swing.) produced by electrofusion. *HortScience* 77:125-130.
- Saunt, J. 1990. Citrus varieties of the world. Sinclair International Ltd. Norwich, England. 126 pp.
- Schubert, T. S., S. A. Rizvi, X. Sun, T. R. Gottwald, J. H. Graham and W. N. Dixon. 2001. Meeting the challenge of eradicating citrus canker in Florida-again. *Plant Disease* 85:340-356.
- Sharma D.R., R. Kaur, and K. Kumar. 1996. Embryo rescue in plants- a Review. *Euphytica* 89:325-337.
- Sinclair, W. B. 1984. The biochemistry and physiology of the lemon and other citrus fruits. University of California, Division of Agriculture and Natural Resources, Oakland, California pp.946.
- Soares Filho, W. S., J. E. Vasquez, M. A. P. da Cunha, A. P. da Cunha Sobrinho, and O. S. Passos. 1992. Degree of polyembryony, size and survival of the zygotic embryo in citrus. *Proceedings of the International Society of Citriculture* 135-138.
- Soost R. K., and J. W. Cameron. 1980. 'Oroblanco', a triploid pummelo-grapefruit hybrid. *HortScience* 15:667-669.
- Soost R. K., and J. W. Cameron. 1985. 'Melogold', a triploid pummelo-grapefruit hybrid. *HortScience* 20:1134-1135.

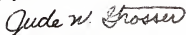
- Soost, R. K., and J. W. Cameron. 1969. Tree and fruit characters of Citrus triploids from tetraploid by diploid crosses. *Hilgardia* 39:569-579.
- Soost, R. K., and M. Roose. 1996. Citrus. IN: J. Jules, and J. N. Moore (eds.) *Fruit breeding: tree and tropical fruits* Vol. 1:257-323. John Wiley and Sons Inc. New York.
- Stall R. E. 1988. Canker. IN: Whiteside J. O., S. M. Garnesny and L. W. Timmer. *Compendium of citrus diseases* 6-7. APS, St. Paul, Minnesota.
- Stall R. E., G. M. Marco, and B. I. Canteros de Echenique. 1982. Importance of mesophyll in mature-leaf resistance to canker of citrus. *Phytopathology* 72: 1097-1100.
- Starrantino, A. 1992. Use of triploids for production of seedless cultivars in Citrus improvement programs. *Proceedings of the International Society of Citriculture* 1:117-121.
- Starrantino, A., and G. Reforgiato Recupero. 1981. Citrus hybrids obtained *in vitro* from 2X females x 4X males. *Proceedings of the International Society of Citriculture* 1:31-32.
- Sugiura, A., T. Ohkuma, Y. A. Choi, R. Tao, and M. Tamura. 2000. Production of nonaploid ($2n = 9x$) Japanese persimmons (*Diospyros kaki*) by pollination with unreduced ($2n = 6x$) pollen and embryo rescue culture. *Journal of the American Society for Horticultural Science* 125:609-614.
- Sukno, N., J. Ruso, C. C. Jan, J. M. Melero Vara, and J. M. Fernandez Martinez. 1999. Interspecific hybridization between sunflower and wild perennial *Helianthus* species via embryo rescue. *Euphytica* 106:69-78.
- Swingle, W. T. 1967. The botany of citrus and its wild relatives. IN: W. Reuther, H. J. Webber, and L. D. Batchelor. *The citrus industry*. Vol. 1:199-422. University of California, Berkeley.
- Swisher, H. E., H. Lavera, and H. Swisher 1977. Lemon and lime juice processing. IN: Steven, N., Shaw, P. E. and Veldhuis, M. K (eds.). *Citrus Science and Technology* 253-289. AVI Publishing Company, INC. Westport, Connecticut.
- Sykes, S. R., and Lewis, W. J. 1996. Comparing Imperial mandarin and Silverhill satsuma mandarin as seed parents in a breeding program aimed at developing new seedless citrus cultivars for Australia. *Australian Journal of Experimental Agriculture* 36:731-738.
- Tusa, N., J. W. Grosser, and F. G. Gmitter. 1990. Plant regeneration of 'Valencia' sweet orange, 'Femminello' lemon, and the interspecific somatic hybrid following protoplast fusion. *Journal of the American Society for Horticultural Science* 115:1043-1046.

- Tusa, N., J. W. Grosser, F. G. Gmitter Jr., and E. S. Louzada. 1992. Production of tetraploid somatic hybrid breeding parents for use in lemon cultivar improvement. *HortScience* 27:445-447.
- Tusa, N., L. Abbate, S. Ferrante, S. Lucretti, and M. T. Scarano. 2002. Identification of zygotic and nucellar seedlings in *Citrus* interploid crosses by means of isozymes, flow cytometry and ISSR-PCR. *Cellular and Molecular Biology Letters* 7:703-708.
- Tusa, N., S. Fatta del Bosco, F. Nigro, and A. Ippolito. 2000. Response of cybrids and a somatic hybrid of lemon to *Phoma tracheiphila* infections. *HortScience* 35:125-127.
- Tusa, N., S. Fatta del Bosco, L. Nardi, and S. Lucretti. 1996. Obtaining triploid plants by crossing citrus lemon cv. 'Femminello' 2N x 4N allotetraploid somatic hybrids. *Proceedings of the International Society of Citriculture* 1:133-136.
- Valdez, J. G., and S. Ulanowski. 1997. *In vitro* germination of stenospemic seeds from reciprocal crosses (*Vitis vinifera* L.) applying different techniques. *Vitis* 36:105-107.
- Vardi, A. 1977. Isolation of protoplast in citrus. *Proceedings of the International Society of Citriculture* 2:575-578.
- Vardi, A. 1992. Conventional and novel approaches to citrus breeding. *Proceeding of the International Society of Citriculture* 39-43.
- Wakana, A., M. Iwamasa and S. Uemoto. 1981. Seed development in relation to ploidy of zygotic embryo and endosperm in polyembryonic citrus. *Proceedings of the International Society of Citriculture* 1:35-39.
- Yamashita, H., I. Shigehara, and T. Hanieda. 1998. Production of triploid grapes by in ovulo embryo culture. *Vitis* 37:113-117.
- Young, R.H. 1986. Fresh fruit cultivars. IN: W. F. Wardowski, N. Steven and G. William (eds.). *Fresh citrus fruits*. 101-142. AVI. New York, New York.
- Zubrzycki H. M. and A. Diamante de Zubrzycki. 1981. Resistance to *Xanthomonas campestris* pv. *citri* (Hasse) Dowson in oranges (*Citrus sinensis* (L.) Osbeck). *Proceedings of the International Society of Citriculture* Vol. 1: 405-409.

BIOGRAPHICAL SKETCH

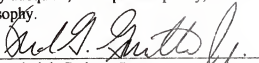
Zenaida Josefina Viloria Villalobos came from Maracaibo, Venezuela. She obtained an Engineering degree in Agricultural Sciences from the Universidad de Zulia. After graduation, she worked as a research assistant in acarology, followed by an assistant professorship teaching plant propagation techniques in the Department of Botany at the same university. In 1997, she was awarded a scholarship from the Universidad de Zulia and the FONACIT (Fondo Nacional de Ciencias.y Tecnologia) to pursue her doctorate degree. Zenaida will return to Maracaibo to reassume her position.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



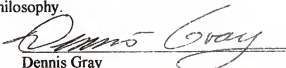
Jude Grosser, Chair
Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



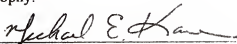
Frederick Gmitter, Jr.
Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Dennis Gray
Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Michael Kane
Professor of Horticultural Sciences

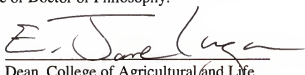
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Renee Goodrich
Professor of Food Science and Human
Nutrition

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2003



Dean, College of Agricultural and Life
Sciences

Dean, Graduate School